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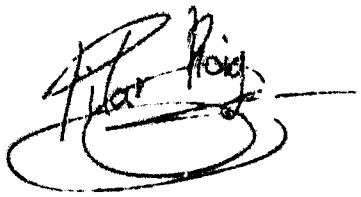
A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, for the Degree of Master of Science.

Johannesburg, February 1986

Declaration :

I hereby declare that this dissertation is my own unaided work.

It is being submitted to the University of the Witwatersrand for the degree of Master of Science and has never been submitted to any other university for any degree or examination.



Pilar Roig Farran

Johannesburg

February, 1986.

ABSTRACT

Several human cytomegalovirus (HCMV) induced proteins have been found in human embryonic lung fibroblast cells (HEF). Monospecific antibodies against three of the glycoproteins (54Kd, 62Kd and 90Kd) raised in rabbits were used to purify the different glycoproteins from HEF membrane preparations through affinity chromatography columns. The optimal conditions for the membrane preparation and the affinity chromatography columns were determined.

Sodium deoxycholate was found to be the most suitable for the membrane solubilization. While a high pH elution buffer gave better yields. The resulting glycoproteins were subsequently separated on polyacrylamide gel electrophoresis and their molecular weights determined as well as their reactivity to the specific antisera on Western blots. Affinity chromatography can be used to purify relatively large amounts of antigen which can induce the production of monospecific antibodies in rabbits. The role of the 54Kd, 62Kd and 90Kd glycoproteins in obtaining a suitable vaccine against human cytomegalovirus is discussed.

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LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
BSA	Bovine Serum albumin
CMC	Critical Micelle Concentration
CMV	Cytomegalovirus
D.O.C.	Deoxycholate
EDTA	Ethylene diamine-tetraacetic acid
EMEM	Eagle's minimal essential medium
FCS	Foetal calf serum
FMA	Fluorescein Mercuric acetate
HCMV	Human Cytomegalovirus
HEF	Human Embryonic Fibroblast
HMEM	Hanks minimal essential medium
HSV	Herpes Simplex Virus
IgG	Immunoglobulin G

Kd	Kilodaltons
MEM	Minimal essential medium
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PEG	Polyethylene glycol
PFU	Plaque forming units
PMSF	Phenylmethylsulphonyl fluoride
SDS	Sodium dodecyl sulfate
TEMED	Tetramethylethylenediamine
Tris	Trishydroxyaminomethane
v/v	Volume per volume
w/v	Weight per volume

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1.0 INTRODUCTION

1.1 CYTOMEGALOVIRUS

Human cytomegalovirus (HCMV) is a Herpes virus capable of producing a variety of clinical conditions in man which may arise either from a primary infection or from the re-activation of latent virus during periods of immunosuppression (Weller,1971; Plummer,1973).

Human diseases associated with cytomegalovirus infection was first recognized by the unique cytopathology induced in host tissue (Jesionek and Kiolemenglov,1904). It was first described (Rowe *et al*,1956; Smith,1956; Weller *et al*,1957) and named "cytomegalovirus" (Weller *et al*,1960) by virtue of the cell hypertrophy which it induces. Since then, many strains of human CMV have been isolated (Michelson-Fiske,1977).

It is composed of a linear, double stranded DNA molecule, approximately $150x10^6$ in molecular weight (De Marchi *et al* ,1978; Geelen *et al*,1978; Kilpatrick and Huang,1979), that is replicated and packaged into an icosahedral capsid within the nucleus of the infected cell, and surrounded by an envelope most probably acquired as the nucleocapsid "buds" through the nuclear membrane into the cytoplasm (Morgan *et al*, 1959; Kanich and Craighead,1972; Iwasaki *et al*,1973; Smith and De Harven,1973).

CMV is almost identical in structure to Herpes Simplex virus (HSV) (Smith and Rassmussen,1962; Goodheart *et al*,1964; Phan Huu Trung and Lardemer,1972). Particles have an icosahedral form with 162 capsomeres separated by a 175 A interspace and with holes 40 µm in diameter (Smith and Rassmussen, 1962). Most particles appear to have incomplete cores and

the ratio of infectious virus to non-infectious virus is in the order of $1/10^6$ to $1/10^8$ (Plummer and Benyesh-Melmick, 1964).

Particle density is $1.18 g/cm^3$ in sucrose gradients (Chambers *et al*, 1971). CMV DNA has a density of $1,716 g/cm^3$ in caesium chloride gradient (Huang *et al*,1973; Huang and Pagano, 1974), the C+G% value in moles percent being between 56-58% with only a few exceptions (Michelson-Fiske, 1977).

There are some discrepancies about the antigenic relatedness of various isolates of human cytomegalovirus. Whereas some results with cross-neutralization test indicate that the different isolates of the virus are unrelated, complement fixation tests and cross-neutralization in the presence of added complement suggest that various HCMV are serologically related (Gupta *et al*, 1977). Studies on the cRNA-DNA hybridization relatedness between different isolates, cRNA-DNA cohybridization *in situ* and DNA-DNA reassociation have demonstrated that all human strains share at least 80% of the DNA of the AD169 strain of CMV (Michelson-Fiske,1977). Moreover, restriction map patterns of some eleven clinical isolates demonstrate considerable diversity of different isolates (Kilpatrick *et al*,1976; Weststrate *et al*, 1983).

For clinical diagnosis of CMV, the virus can be isolated from infected new borns who excrete large amounts in urine. CMV infected adults, however, seldom shed sufficient virus for isolation (Pereira *et al*,1982). In most cases, serological techniques which depend on showing a fourfold rise in antibody titer from the acute to convalescent sera are used for diagnosis (Cremer *et al*,1978).

Clinical isolates of CMV characteristically grow slowly in cultured fibroblasts. Since little extracellular infectious virus is produced by these cells, newly recovered strains frequently are difficult to maintain and passage in the laboratory. In contrast, CMV which has been serially

passaged over extended periods of time often multiplies readily and produces relatively large amounts of infectious intra- and extracellular virus (Kanich and Craighead, 1972). Among the characteristics originally ascribed to cytomegalovirus were its great host and tissue specificity. However, both these characteristics have been questioned (Plummer and Goodheart, 1974; Michelson-Fiske *et al*, 1975; Knowles, 1976).

Human CMV can to some extent infect a number of non-human cells. It can enter monkey Vero cells (Waner and Weller, 1973; St. Jeor *et al* 1974; Waner and Weller, 1974), Bovine cells, mouse fibroblasts (Gonczel *et al*, 1975) and hastly hamster cells (Albrecht *et al*, 1973). In these cells, host and virus DNA synthesis is stimulated (St Jeor *et al*, 1974) but co-cultivation with human fibroblasts is usually required (Waner, 1973) to rescue infectious virus.

In vitro, the virus has only been found to replicate in fibroblasts and myometrial cells. However, *in vitro* infection of human CMV in human lung epithelial cells (Michelson-Fiske *et al*, 1975), human embryonic (St Jeor and Rapp, 1973a; St Jeor and Rapp, 1973b) and adult kidney cells (Michelson-Fiske, 1977) and thyroid cells (Knowles, 1976) have been realized. Human CMV may also replicate in B lymphocytes, though succesful infection is apparently dependant on the presence of an EBV genome (Huang and Pagano, 1975).

Several authors have looked for specific proteins in CMV infected cells (Savor and Agaby, 1975; Gupta and Rapp, 1977; Gupta *et al*, 1977). Some 32 polypeptides have been found by SDS-PAGE with a molecular weight ranging from 13500 to 235000 kd. of which some are reported to be glycosylated. However there is still no consensus as to how many proteins comprise the HCMV virion and what their characteristics are. Many of these proteins appear to be associated with virus and virus-related structures.

Indeed, studies to investigate the role of proteins appearing during the infection process with this virus have resulted in the observations that four types of virus particles can be recovered from infected cells. These particles have been distinguished by Gibson (1981) on the basis of their intracellular compactimentation, sedimentation properties in rate-velocity sucrose gradients, protein composition and infectivity.

By analogy with the apparent counterparts in Herpes Simplex Virus infected cells, these particles have been designated as A-, B-, C- capsids and virions. A- capsids, compose of three protein species, have the simplest structure virions containing protein species are structurally the most complex (Schramm, 1985).

Further dense bodies are found in CMV-infected cells these being aggregates of virus substructures that are surrounded by a common envelope and are abundant in the cytoplasma of infected fibroblasts. They are similar in composition to virions, but not identical (Forghani and Schmidt, 1980). Experimentally, virions are difficult to separate from dense bodies for analysis. Various results have been reported, as the starting material used by different workers is heterogeneous and different methods of analysis have been employed.

It is well established that productive human cytomegalovirus infection results in alterations in the morphology, social behavior and antigenicity of human fibroblasts. Morphological changes in cytomegalovirus and Epstein-Barr virus infected cells have been extensively described in electron microscopy studies (Falcieri *et al*, 1980). Documented changes include cell rounding and hypertrophy (Michelson-Fiske, 1977), decreased cellular adhesiveness (Diosi *et al*, 1972) altered surface topography (Garnett, 1979a), fusion phenomenon (Booth *et al*, 1978; Garnett, 1979b) and altered antigenicity at early stages of infection (The and Langenhuysen, 1972).

These observations suggest that alterations in the protein composition of the plasma membrane may occur during the virus infection cycle, despite previous suggestions that HCMV does not mature at the outer cell surface (Severi *et al*, 1979). Insertion of HCMV-specified glycoprotein into the plasma and microsomal membranes of HCMV-infected cells has been reported at 20-24 hrs after infection (Stinsky *et al*, 1979). Also, it has been shown that the neutralizing antibodies raised against HCMV virions react with the surfaces of infected cells (Fereira *et al*, 1982b), which suggest that some glycoproteins exposed on HCMV-infected cells are antigenically related to the envelope components of the virus (Stinsky, 1976; Farrar and Oram, 1984).

An analysis of the plasma membrane proteins of human embryonic fibroblast infected with CMV strain AD-169 was performed using *in vitro* radioactive labelling techniques followed by PAGE by Sullivan-Tailyour (1984) of the twelve virus-induced proteins detected in infected cells, nine were glycoproteins of which glycoproteins 34kd, 52-54 kd, 60-63 kd, 70-72 kd, 98-103 kd and 145-150kd and proteins of 130-133 kd and 260-270 kd were considered significant. The 60-62 kd, 70-72 kd and 130-133 kd components were detectable at early stages of infection (8hrs), while the others only appeared in the membrane from 48hrs to 80hrs after infection. Serological studies indicated that the 34kd, 70-72 kd, 98-103 kd and 145-150kd components may be HCMV-specified virions constituents, as these glycoproteins reacted with antibodies raised against virions and extracted envelope glycoproteins

Of immunological importance was the exposure on the cell surface of the proteins moieties of 70-72 kd and 130-133 kd proteins at 8hrs and 53-55 kd, 61-63 kd, 70-72 kd and 145-148kd components at 80-90 hrs after infection. Pooled human immune sera contained antibodies which reacted with these exposed proteins, as well as with three other virus induced membrane components of 230-240 kd, 98-100 kd and 78-80 kd.

Since HCMV is a highly cell-associated virus it is believed that destruction of virus-infected cells is of prime importance in immunity. Antibodies to virally-coded membrane antigens would presumably play a major role in the destruction of infected cells by antibody-dependent cell-mediated cytotoxicity (ADCC) reactions. Indeed, if virus-specific constituents present in both the virion and plasma membrane of HCMV-infected cells do elicit neutralizing or cytotoxic antibodies they would be suitable candidates for the development of a subunit CMV vaccine. This study has revealed that virus-induced glycoproteins of 145-150 kd, 70-72 kd, 60-62 kd and 53-55 kd are inserted into the plasma membrane of HCMV-infected cells, are exposed at the surface and do elicit specific antibody production in infected individuals. The isolation of these glycoproteins may therefore be of value for the production of an effective subunit vaccine. Furthermore, cloning of the viral genes which code for such glycoproteins and their expression in a suitable eukaryotic cell would similarly contribute to vaccine development (Sullivan-Tailyour, 1984)

1.2 PLASMA MEMBRANES

The plasma membrane surrounding living cells plays a role in intracellular metabolism, but what distinguishes its structure from other organelles is that the plasma membrane also mediates interactions between the cell and its external environment,. The simplest of these interactions maintain a desirable intracellular milieu by bringing in needed substances and getting rid of waste products. The plasma membrane acts as a passive diffusion barrier to charged and large molecules, and in addition carries out facilitated diffusion, active transport, endocytosis (pinocytosis and phagocytosis) and exocytosis. Further, this membrane is involved in

locomotion and chemotaxis, processes which could remove a cell from noxious surroundings or to a more nutritive medium. ON a more complex level, cells must communicate with other cells; this is especially important in the formation and maintenance of multicellular organisms. The plasma membrane functions in the secretion and reception of hormones, conduction of nerve impulses and direct cellular interaction such as adhesion and contact inhibition. Finally on yet another level, the plasma membrane may be involved in such sophisticated processes as immunological defence and information storage and retrieval. (De Pierre and Karnovsky, 1973).

The progress made in developing techniques for isolating plasma membranes is slow, due largely to the considerable complexities inherent in working with membranes of any sort. Inherent problems associated with the development of plasma membrane purification methods include :

- Cell disruption - The plasma membrane may be converted into "ghosts", open envelopes, large and sometimes specialised sheets , or small vehicles. A criterion for their isolation is necessary. Two main techniques are available :

  - Physical Methods - These rely on stress distortion of vulnerable membrane regions. This includes sonic irradiation and mechanical homogenization, which presumably act via liquid shear, osmotic stress, which extends the surface, and low ionic strengths, electrostatic interactions among fixed membrane charges which can cause local disruptive distortion.

    Some of the methods disrupt nonselectively and due to the local heat and free radicals are very likely to disturb membrane structure causing nuclear, lysosomal, mitochondrial as well as

plasma membrane damage. Such methods are sonication and high liquid shear devices such as the French press (Wallach, 1972).

The dounce type homogenizes are widely used, but shearing depends on the clearance and the relative motion between pestle and mortar which is difficult to control (Wallach, 1972).

Rapid decomposition of an inert gas , selectively ruptures surface membranes. Upon return to normal pressure, gas bubbles form at cell surfaces and disrupt these by local shear. Provided this is done under controlled, quantitative, iso-osmotic conditions, in an inert atmosphere, then problems of local heating and/or rapture of nuclei, mitochondria, or lysosomes are avoided.

The ionic, osmotic and colloid composition of the media for cell disruption is a crucial factor

- Chemical Method - Chemical cell disruption, using hypotonic or hypo-ionic media, pH extremes, surface-active agents, lipases or proteases are generally quite indiscriminate in their action and therefore are used only in special instances.

- Membrane recovery

  - The fate of the plasma membrane is often difficult to monitor, but this depends on the method used for isolation. Many different methods are available throughout the literature that meet every necessity. For the fragmentation of plasma membrane, again the degree of fragmentation can be related to the procedure used. Physical properties of the surface fragments often overlap those of other organelles.

- Markers - To safely be able to study the plasma membranes for the purpose for which they were isolated it is essential to determine the purity of the preparation. In the initial isolation there are two means of following the desired organelle :

  - Morphological markers

  - Enzymatic markers (Wallach, 1972)

Based on the above principles various procedures have been published.

In 1965 Warren and Glick developed a method for plasma membrane isolation. This method involved the treatment of the cells with substances such as fluorescein mercuric acetate (FMA), which stabilized the plasma membrane so that the plasma membrane could be isolated. However, the cells growth in monolayer first had to be converted to a single cell suspension through a variety of methods

In 1970, Barland and Schroeder published a modified FMA technique in which the stabilized plasma membranes were stripped from cells while the cells remained attached to the growing surfaces. This avoids the possibility of artefact formation (Quissel *et al*, 1977). Today, this technique is used in L cells, fibroblasts, Balb/c cells, etc. The advantages of which are as follows :

- Simple

- Rapid (less than 2 hours).

- Avoids the necessity for elaborate equipment

- Less risk of losses during transfer

- Minimal bacterial contamination

- Homogeneous membrane preparation yield

- No Trypsin is used

However several authors criticize the technique, remarking a number of disadvantages :

- Only a selective portion of the plasma membrane is isolated (McClure *et al*, 1979).

- Cytoplasmic elements might adsorb to the plasma membrane (De pierre and Karnovsky, 1973)

However, Sullivan-Tailyour (1984) showed that membranes obtained from fibroblasts by this technique were clear.

Brunette and Till (1971), adapted the aqueous two phase polymer separation system for the isolation of L-cell plasma membranes. The separation of soluble materials by differential partition between two immiscible phases is one of the classical purification procedures. Aqueous solution systems, can be buffered and rendered isotonic, proving useful for the separation by counter-current partition and distribution of cells, particles and membranes.

The advantages are numerous :

- Sensitive

- Rapid (less than two hours)

- Favourable yield of membrane preparation
- Mild
- Flexible (Can vary with pH, ionic strength and polymer composition).

Scott in 1971 reported a new technique for the isolation of plasma membranes which involved vesiculation of the plasma membrane, based on the observation that a variety of aldehydes and disulfide blocking agents promote the formation and release of plasma membrane vesicles from cells in culture. The technique has been modified and improved with a variety of compounds today available. Its advantages are :

- No significant cytoplasmic contamination
- Antisera studies reveal that the vesicles contain at least certain antigens that are represented over the entire membrane.

There are quite a number of reports in the literature which involves rupture of the cell, followed by separation of the plasma membranes from the homogenate by physical techniques. These methods tend to describe in detail the composition of the homogenization medium, the type of homogenizer used, the clearance between the pestle and the homogenization vessel, the speed of the pestle, the number of strokes applied and the extent to which the homogenate should be diluted after disruption is completed in order to set repeatable conditions. However, a number of disadvantages have been reported :

- Complex
- Lengthy

- Membrane yield is low

Other authors use a hypotonic medium which does attempt to disrupt the cells by means of osmotic forces, thereby reducing the amount of mechanical force (Schramm, 1985).

One of the most significant dangers involved in homogenization is the exposure of the plasma membrane, when cells are broken, to enzymes to which they are normally not accessible.

1.3 AFFINITY CHROMATOGRAPHY

Many workers throughout the literature have reported the use of affinity chromatography as a tool to purify virus antigens. Rasmussen *et al* (1985) used immunoaffinity chromatography to isolate virus polypeptides of HCMV. Eisenberg *et al* (1982) used the same technique to purify a glycoprotein of Herpes Simplex Virus. Wroblewska *et al* (1985) describes a procedure to purify glycoproteins from Varicella Zoster virus.

Affinity chromatography is used to purify one of the components of a system consisting of two or more species whose reversible interactions reflect affinity with a high degree of biological specificity (Cuatrecasas and Anfinsen, 1971a). The term was originally used to describe the simples cases of such interacting systems : those constituted of enzymes and competitive inhibitors (Cuatrecasas *et al*, 1968). However, the potential application of this kind of functional purification to a variety of other kind of complexing systems was immediately apparent (Cuatrecasas, 1971b).

A number of different methods for coupling to inert supports have been developed recently and are discussed in the review by Venter (1982). When preparing the affinity gel, decision has to be made about the nature of the ligand which will be coupled to the inert support. This could be the substrate or an inhibitor in the case of an enzyme, an antibody in the case of a membrane bound antigen, a hormone or its antagonist in the case of a receptor. Conditions of coupling are crucial, as they should not modify the nature of the interaction between the ligand and the membrane protein (Strosberg, 1984).

Several methods have been described to immobilize antibodies on solid inert matrices: Bromo acetylation and coupling to cellulose and other less used techniques were gradually replaced by cyanogen bromide-activated sepharose. Gersten and Marchalonis (1978) analysed the problems of this method and offer possible solutions.

After coupling the ligand to the inert support and assessing the amount of fixed compound per millilitre of gel comes the crucial moment of evaluating the biological properties of the immobilized ligand. Venter (1982) discussed various methods to analyse the affinity gel prior to the purification step.

Affinity chromatography depends on a variety of chemical interactions : electrostatic, van der Waals, and hydrophobic. These occur between proteins or peptides, carbohydrates, lipids and synthetic compounds. Aqueous solvents are usually used. This is not necessarily true when membrane proteins are to be purified. First of all, their solubilization often requires detergents which cannot be easily removed without loss or precipitation of the proteins. Second membrane proteins are probably asymmetric, with a more hydrophobic part turned in toward the membrane and a more hydrophilic part turned toward the exterior of the cell. While the hydrophobic part will be of interest in hydrophobic affinity

chromatography, the carbohydrates may serve for lectin affinity chromatography (Strosberg, 1984).

Purifications of membrane proteins stands or falls with a good solubilized preparation of membrane proteins. No general methods can be prescribed since each cell type has membranes of different composition and thus presents specific difficulties. Solubilisation of the membrane proteins can be carried out with one of various detergents, ionic or non-ionic : Triton X-100, Lubrol PX, Nonidet P-40, Sodium dodecyl sulphate, digitonin, Sodium Deoxycholate ...

It is important to keep in mind that the lipids and proteins of a native membrane interact with each other in a complex fashion that will differ in detail from membrane to membrane. Therefore, for example, no detergent can be singled out as most effective for membrane solubilization. Similarly some protein structures are more sensitive to their environment than others, and the ability to tolerate some mildly disruptive detergents without loss of activity is quite variable. Quite generally, the optimal detergent for a particular membrane protein has to be found empirically and may depend on the type of experiment one wants to do. (Helenius *et al*, 1983)

Some important characteristics of detergents are :

- **Critical Micelle Concentration (CMC)**

  In aqueous solutions detergent molecules occur in the form of monomers and micelles. The micelles are fairly monodisperse compact aggregates where the apolar groups of the detergent molecules are sequester into the centre and the polar groups face outwards. For practical purposes the CMC represents the highest monomeric detergent concentration (and thereby the highest detergent chemical potential) obtainable.

- **Micelle size**

  The micelle size increases and the CMC decreases with increasing size of the apolar moiety of the detergent molecule and, to a lesser extent, with decreasing size and polarity of the polar groups.

- **Partial specific volume (V)**

  This is important because the buoyancy factor ($1-V\rho$) determines the rate of sedimentation at any density ρ.

Different detergents

- **Alkyl Ionic Detergents**

  These detergents denature the proteins at the concentration and temperatures used. They also usually dissociate complex proteins into their constituent polypeptide chains. Sodium dodecyl sulphate is the standard.

  Since proteins differ in their sensitivity to these detergents, they can be used in selective isolation.

  Their properties are strongly affected by the ionic strength and by the nature of the counterion. Temperature also plays an important role. (Helenius *et al*, 1976)

- **Nonionic Detergents with Polyoxyethylene or Sugar Head Groups**

  They do not usually denature proteins. They are efficient in breaking lipid-lipid and lipid-protein interaction in membranes, but ineffec-

tive when it comes to breaking interactions between proteins. Triton X-100 is one of them. (Helenius *et al*, 1976)

- **Lysophospholipids**

When added to cells, they cause lysis and cell fusion, and at a higher concentration they can solubilize membranes (Gent *et al*, 1964; Poole *et al*, 1970).

There are reasons to believe that they are especially mild in their effects on sensitive membrane proteins, but it has yet to be proven.

- **Bile Salts**

They differ from the detergents described so far in structure and in detergent properties.

Deoxycholate and cholate are the most commonly used. The free monomer concentration (and thus the chemical potential) for bile salt detergents increases considerably even after the CMC has been reached, and additional effects on membrane proteins can sometimes be observed (Helenius *et al*, 1976)

As well as the alkyl ionic detergents, the bile salts are sensitive to conditions such as temperature or pH, and hence the choice of buffer conditions is therefore very important.

Most of the dihydroxy bile salts are more powerful membrane solubilizers than the trihydroxy bile salts and most nonionic detergents. Furthermore, they tend to dissociate interactions between proteins more effectively. However, high concentrations of

deoxycholate have in fact been shown to inactivate several membrane enzymes (Helenius *et al*, 1983)

The maximal capacity of the affinity gel is determined by applying increasing amounts of solubilized membranes until the column is saturated. In studying the absorptive qualities of the gel it is often crucial to determine the ratio of specific to nonspecific adsorbed protein : It is this ratio that will best identify opportunities for purification.

Some method of evaluating the amount of active protein applied to the gel must be available in order to measure the efficiency of absorption and elution procedures. Usually one uses the binding capacity expressed in picomoles of binding protein per milligram of membrane protein.

In the case of antibody/antigen columns, either one of these two entities is coupled and the other is retained. Elution is generally by nonspecific procedures such as high salt concentrations or low pH. The immunoadsorption procedure was initially used primarily to purify antibodies using the insolubilized antigen. In some cases, fragments of the antigen are available in sufficient quantities to induce the formation of specific antibodies and these are then used to purify more antigen.

Affinity chromatography is a powerful tool through which relatively large amounts of antigen in an undenaturated form can be purified. Furthermore, the purity of the resulting antigen is high enough to raise monospecific antibodies once injected into animals. This is an alternative methods to the monoclonal antibodies which preparation is time-consuming and tedious.

It appears that the most productive approach to isolate and purify membrane proteins which are not denaturated involves three steps : Suitable solubilisation of purified plasma membranes, affinity chromatography of

solubilized ligand using an antibody which may have been prepared from proteins extracted from gel slices, and finally immunoaffinity purification (Strosberg, 1984)

Electrophoretic molecular sieving in detergent laden polyacrylamides also has become a key method for the analysis of membrane proteins. Through the driving force of an electric potential most of the complexes fractionate in polyacrylamide of appropriate porosity, according to size. The polyacrylamide gels are calibrated under identical conditions, with proteins of known molecular weight, allowing the determination of molecular weights of unknown peptide chains to approximately 3% (Wallech, 1972).

Although SDS-PAGE only provides information of the size and amount of protein molecules, assays using specific antibodies as a detecting probe can be used to greatly facilitate the identification and characterization of individual membrane proteins. These assay procedures are based on the transfer, either by electrophoresis or by diffusion, of proteins, from the polyacrylamide slab gel to an insoluble matrix, such as commercially available nitrocellulose sheets. Such immobilized proteins are then readily accessible to antibodies or other specific probes; this makes possible the *in situ* localisation and characterisation of individual proteins in complex mixtures (Haid and Suissa, 1983).

The aim of this project was to determine the optimal conditions to purify three human cytomegalovirus infected human embryonic lung fibroblast cell membrane glycoproteins (54Kd, 62Kd and 90Kd) through immunoaffinity chromatography columns.

These conditions include cell disruption, membrane solubilization and elution procedures from the immunoaffinity chromatography columns. The different preparations were then analysed on polyacrylamide gels.

The actual role of immunoaffinity chromatography towards a future vaccine against CMV is discussed.

2.0 MATERIALS AND METHODS

2.1 CELLS

Human embryonic lung fibroblast (HEF) were obtained from stock cultures provided by the University of the Witwatersrand, Johannesburg. Stock cultures were grown as monolayers in 250 cm^2 plastic tissue culture flasks (Costar, U.S.A.) at 37°C. The growth medium used was Eagle's minimal essential medium (EMEM) (Gibco, U.K.) with 100 µg of streptomycin and 100 U of penicillin per mℓ, and supplemented with 10% foetal calf serum (FCS) (Gibco, U.K.) for growth and 2% FCS for maintenance. Cells were fed every two to three days and when 80% confluent, they were subcultured.

The cells were subcultured at 4-6 day intervals. The maintenance medium was decanted and replaced with phosphate buffer saline pH 7.0 ("Appendix 1" on page 68) which was gently swirled around the flask and decanted. This was followed by treatment with enough trypsin-ethylenediamine tetraacetic acid (EDTA) solution (See "Appendix 1" on page 68) to cover the monolayer, and the culture flask incubated at 37°C until the monolayer detached from the tissue culture flask substrate. Hanks minimal essential medium (HMEM) (Gibco, U.K.) was added to neutralize the effect of the trypsin on the cells in suspension. McCartney bottles (20 mℓ) were used to pellet the cells at 1000 rpm for 5 minutes in a Sorvall swing-bucket rotor centrifuge. Cells were washed in 10 mℓ HMEM, centrifuged again, and resuspended in sufficient growth medium (EMEM) supplemented with 10% FCS. Two flasks were inoculated with the yield of one. The cells were used for experimentation between the fifth and the twenty fifth transfer.

Tissue culture flasks were placed into an 5% CO_2 in air incubator at 37°C with their caps loosened. After two hours the caps were tightened again and the flasks left at 37°C.

For storing purposes, confluent HEF cells were trypsinized following the above procedure, and pellet resuspended in medium consisting of 50% MEM, 40% FCS and 10% glycerol (v/v). This was dispensed into Nunc (Nunc, Weil) plastic tubes and kept frozen at -70°C. When required, the cells were thawed rapidly at 37°C, diluted with MEM and pelleted. The cells were resuspended in 10 mℓ fresh growth medium and plated into one 25 cm^2 plastic flask to allow attachment.

2.2 INFECTION OF HEF CELLS

The human cytomegalovirus (HCMV) strain used was AD169, obtained from ATCC (American type culture centre, U.S.A.), which had been serially passaged to yield a high titer stock. In order to make a virus stock, a vial of virus was diluted conveniently with EMEM. The diluted virus was poured onto a HEF monolayer of 80% confluency which had been washed free of serum containing EMEM using PBS, pH 7.0. The virus was allowed to adsorb for 60-90 minutes at 37°C and then decanted. The cells were then fed with EMEM containing 2% serum.

Cells were fed every 2 days with maintenance medium, and when extensive cytopathic effects were observed (Approximately 5 days), they were harvested by scraping the infected cells free from the substrate using a rubber policeman. An equal volume of 70% sorbitol (w/v) was added and the virus stock stored at -70°C in 1 mℓ aliquots. Before use, the stock was

freeze-thawed to lyse the cells present and release cell-associated virus.

A tissue culture infectivity assay was performed to determine the approximate titer of the virus stock. Nearly confluent monolayers in 24 well trays were inoculated with serial dilution of the stock. After 5 or 6 days of infection , the infectious dose fifty (ID_{50}) was taken as the highest dilution which gave an observable cytopathic effect in 50% of the wells. From this the $TCID_{50}$ the virus titer was estimated

When infected cells were required for experimentation, a stock of virus diluted to 1×10^7 with EMEM was added to a flask of HEF of 80% confluency, which had been thoroughly washed with PBS, pH 7.0. The final virus : cell ratio was 5:1. After 60-90 minutes adsorption time, the virus was removed and maintenance medium added. Cells were fed every 2 days with maintenance medium as mentioned above, until extensive cytopathic effects were observed, after which they were utilised.

2.3 MEMBRANE ISOLATION AND SOLUBILIZATION

Plasma membrane were isolated by the method of Krah and Crowell (1982).

CMV-infected and uninfected HEF monolayers were treated with 50 mM EDTA and the suspended cells were centrifuged at 1000 rpm for 15 minutes. Pelleted cells were washed several times with 10 mℓ of phosphate buffer, pH 7.0. Different concentrations of phosphate buffer were used in order to find the best lysis buffer (0.02 to 0.3 M) (See "Appendix 2" on page 70). Cells were allowed to swell for 30 minutes with phosphate buffer, pH 7.0 to a concentration of 4×10^7 cells per mℓ. Cells were disrupted

with 15 to 25 strokes in a 15 mℓ dounce homogenizer. The disruption was monitored examining small fractions of the pellet under a light microscope. All manipulations were performed at 4°C. Once 90% of the cells were ruptured the homogenization was ended. The remaining cells and nuclei were separated through several centrifugations at 800, 1200, 2200 rpm respectively. The supernatant was removed and centrifuged at 25000 rpm for 60 minutes at 4°C in a SWTi55 rotor of the Beckman-LC55 ultracentrifuge.

The pellet was resuspended in solubilizing solution. Two different detergents were used for this purpose; Triton X-100 (0.1%) and Sodium Deoxycholate (0.4%) (See "Appendix 2" on page 70).

The solubilizing solution was allowed to act for 15 to 30 minutes after which it was centrifuged at 30000 rpm for 1 hour in a SWTi55 rotor of the Beckman LC55 ultracentrifuge, and the supernatant stored at -70°C until needed.

To determine the most convenient solubilizing agent SDS polyacrylamide gel electrophoresis (PAGE) was performed, on samples treated with both agents. The effect of dialysing the samples before PAGE was also analysed in the same gel.

2.4 PROTEIN ESTIMATION

The amount of protein was estimated by the coomassie blue reaction (Bio-Rad, U.S.A.). This is a rapid and sensitive method to quantify proteins.

A standard curve was made adding increasing amounts of a standard protein to the dye reagent. The absorbance of the mixture was measured at 595nm after 2 min and before 1 hour against a blank containing 0.1 mℓ of the buffer or salt solution and 5mℓ of the dye reagent.

Aliquots (0.1mℓ) of the membrane solution was mixed with 5mℓ of the dye reagent and the absorbance measured at 595 nm as explained above. This absorbance value was plotted against the standard curve to determine the protein content. (Hanson and Philips, 1981).

2.5 ANTISERUM PURIFICATION

The three antisera raised in rabbits against the three CMV membrane antigens (62kd, 54kd, 90kd) were provided by S. Holmes, University of the Witwatersrand, Johannesburg, and purified through 10mℓ Protein A - Sepharose CL-4B affinity chromatography columns (Pharmacia Fine Chemicals, Uppsala, Sweden) to produce IgG. The column was prepared following the instructions of the manufacturer.

For purification (1mℓ) of crude antiserum was removed from the vial with disposable sterile syringe and needle, and passed through ½ inch glass wool in disposable Pasteur pipette into a 5mℓ graduated cylinder. An equal volume of buffer A (See "Appendix 3" on page 71) was passed through glass wool. The antiserum buffer mixture was carefully applied to the column. The column was washed with 5 to 10 volumes of buffer A and the flow rate adjusted to 1 drop per 15 seconds and 3mℓ samples collected.

The absorbance of the fractions were read at 280nm until the absorbance was near zero. The IgG fraction was eluted off the column with 70mℓ of

buffer B ("Appendix 3" on page 71) and 30mℓ sterile dd water. The flow rate was adjusted to 1 drop per 7 seconds and 3 mℓ samples were collected until the absorbance was near zero. The column was washed with 25mℓ of buffer A and stored at 4°C.

IgG fractions were dialysed against dd water in the cold room for at least 1 hour, and placed in PEG 6000 to concentrate. When concentrated to the desired volume (1-2 mℓ), They were dialysed against PBS buffer overnight (Miller and Stone, 1978; Sibara, 1985).

The protein amount was measured by the Coomassie blue method (Bio-Rad, U.S.A) as seen above and the IgG fractions were stored at -20°C until used.

2.6 ANTIGEN PURIFICATION

2.6.1 PREPARATION OF THE COLUMN

The cyanogen bromide activated sepharose 4B was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Syringes (2.5mℓ) were used as columns.

The amount of protein (IgG) per mℓ of gel was determined in part by the amount of available IgG and was as follows :

- **54Kd** : 1mg IgG per mℓ of gel with a final volume of 3mℓ of gel and 3mg IgG.

- **62Kd** : 1.43mg IgG per mℓ of gel with a final volume of 2.5mℓ of gel and 3.57mg IgG.

- **90Kd** : 0.8mg IgG per mℓ of gel with a final volume of 2mℓ of gel and 1.6mg IgG.

The required amount of CNBr-activated sepharose 4B was washed and swollen for 15 minutes in 1M HCl on a sintered glass filter (Porosity G3).

The gel was washed with coupling buffer (See "Appendix 4" on page 73) (5mℓ per gram dry gel) and transferred to a solution of the ligand. The IgG to be coupled was dissolved in coupling buffer and mixed with the gel suspension in an end-over-end mixer, using a McCartney bottle in a waterbath for 2 hours at room temperature. The gel was transferred to 0.2M glycine as blocking agent for remaining active groups for 2 hours at room temperature.

To remove the excess uncoupled ligand that remained, the adsorbent was washed alternately with high and low pH buffer solutions four times. Acetate buffer, pH 4.0 and coupling buffer, pH 8.3 (See "Appendix 4" on page 73) were used.

Blocking agent was washed away with coupling buffer and the protein-sepharose conjugate column was stored at 4°C with coupling buffer, and 0.02% Sodium azide was added as bacteriostatic agent.

2.6.2 AFFINITY CHROMATOGRAPHY

Two different elution buffers, one of high pH (11.5) and another of low pH (4.0), were used separately (See "Appendix 4" on page 73) and the results compared.

The column was firstly washed with coupling buffer. CMV-infected fibroblasts (HEF) membranes from 5×10^7 cells solubilized in detergent were passed through each column.

In a separate experiment and for comparison purposes, uninfected fibroblast (HEF) membranes from 5×10^7 cells solubilized in the same manner as the CMV-infected cells were also passed through each column.

The protein concentration of CMV-infected fibroblast membranes were 0.43mg/mℓ.

The protein concentration of uninfected fibroblast membranes were 0.56 mg/mℓ.

The fractions collected after passing the sample were passed again through the column. This step was repeated 10 times, so that all the antigens could bind to the antibody of the column. Then the column was washed with coupling buffer to wash away the unbound protein. The absorbance of the fractions were measured at 280nm against coupling buffer as blank. Once the absorbance was near zero the antigen was eluted form the column with the desired elution buffer.

The absorbance of the fractions were measured and the fractions collected until the absorbance against the elution buffer was near zero. Then, the fractions were dialysed against against PBS overnight, concentrated to

100µℓ in a Minicon protein concentrator (Amicon, M.A., U.S.A.) and run on polyacrylamide gels.

2.7 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Protein samples were separated using a modification of the discontinuous polyacrylamide gel system of Laemmli (1970). The slab gels used consisted of a resolving gel of 12% acrylamide (See "Appendix 5" on page 75). A Pharmacia Fine Chemicals vertical slab gel apparatus was used. Pre-electrophoresis was carried out overnight prior to loading the samples at 20mA per gel.

The splitting solution (See "Appendix 5" on page 75) was added to the sample in a ration 1:1 and 100µℓ of the total mixture was added to each well. The wells of the edges were discarded to avoid the problems due to the current effects. Tracking dye was used (See "Appendix 5" on page 75). A molecular weight marker was run in one track of each gel in order to establish the molecular weight of the resulting bands.

Electrophoresis was performed at room temperature for 5-6 hours at 40mA per gel.

The gel was then fixed in 15% trichloroacetic acid overnight (See "Appendix 5" on page 75), followed by overnight staining in Coomassie brilliant blue (See "Appendix 5" on page 75), and destained for 1 to 2 hours in destaining buffer (See "Appendix 5" on page 75)

2.8 PROTEIN BLOTTING

2.8.1 METHOD

Proteins separated by SDS-PAGE gels were transferred onto nitrocellulose paper (Sartorius, Germany) following a procedure initially described by Towbin, Staehelin and Gordon (1979).

A sandwich was prepared as follows :

- a scouring pad (Scoth-Brite)
- two sheets of Whatman filter paper (Whatman, England)
- a nitrocellulose sheet
- the SDS_PAGE gel
- two sheets of Whatman filter paper
- a scouring pad (Scoth-Brite).

All air bubbles between the gel and the nitrocellulose paper were removed. The assembly was placed in a Pharmacia destaining apparatus with a gel nearest the cathode. The electrode buffer (See "Appendix 6" on page 79) was poured into the tank to cover the assembly. The electrophoresis was performed for 3 hours at 0.8-1.0 amp (24-36V).

2.8.2 ANTIBODY TREATMENT

The nitrocellulose paper was incubated at 37°C for 1 hour in a 2% solution of bovine serum albumin (BSA) (w/v) in 10mM Tris-HCl pH 7.4, and 200mM NaCl. The filter was shaken gently to saturate all unbound filter.

The filter was treated with antibody (1:100 dilution in 10mM Tris-HCl pH 7.4) at 37°C for 1½ hours. Then it was washed four times in 10mM Tris-HCl pH 7.4, 5 minutes each time

The sheet was incubated with anti-IgG-peroxidase conjugate (1:1000 dilution in 10mM Tris-HCl pH 7.4) (Cappel, Cochranville, P.A., U.S.A.) at 37°C for two hours, and washed four times as above. It was then incubated with substrate 1,4 dichloronaphtol (25µg/mℓ) (Sigma, St Louis, U.S.A.) plus 0.01% H_2O_2 in 10mM Tris-HCl pH 7.4 for 20 minutes at room temperature for the colour to develop. The sheet was washed with distilled water and dried on filter paper.

3.0 RESULTS

3.1 MORPHOLOGY OF THE CMV-INFECTED AND UNINFECTED HEF CELLS

The morphology of the CMV-infected and uninfected HEF cells were studied under the light microscope (Figure 1 on page 32 and Figure 2 on page 33)

The uninfected HEF cells used as control were fusiform, spindle-shaped, and arranged in an organized parallel monolayer.

Following infection with HCMV, a characteristic sequence of morphological changes took place in HEF cells. Using a virus stock culture of 10^7PFU/mℓ and with and infection of 5-10 PFU/cell, early visible changes occur after 24-48 hours after infection. These were characterized by cell rounding and enlargement.

After 80 hours from infection Cytomegalia was marked. The nuclei became more prominent. Severely infected HEF cells tended also to lose their potential for organized growth.

After this stage the cells began to degenerate. Eventually the infected cells were detached from their substrate and harvest after 5-6 days from infection.

This sequence of events was considered characteristic and only cultures exhibiting such symptoms were used for further studies.



Figure 1. Uninfected Human Embryonic Lung Fibroblast cells used as control: Note the fusiform characteristic morphology of the cells and its parallel disposition. Magnif. 500×



Figure 2. Human cytomegalovirus-infected human embryonic lung fibroblast cells: Note the changes in morphology after 5 days of infection. Magnif. 500×

3.2 MEMBRANE PREPARATION AND SOLUBILIZATION

Phosphate buffer was assessed for its use in cell lysis over the range from 0.0M to 0.3M, 0.2M gave the best result.

In order to find the best detergent to solubilize the membrane preparations, samples were treated with both Triton X-100 (0.1%) and sodium

deoxycholate (0.4%) and the samples were run on polyacrylamide gel to estimate protein solubilization. The result of dialysing the membrane preparation against PBS overnight, was also studied.

The gel was loaded with 20 µg of protein on each well as follows:

- Lane 1 - Low molecular weight marker.
- Lane 2 - HCMV-infected HEF membranes solubilized with sodium deoxycholate, but not dialyzed before PAGE.
- Lane 3 - HCMV-infected HEF membranes solubilized with Triton X-100, but not dialysed before PAGE.
- Lane 4 - Dialysed sample of HCMV-infected HEF membranes solubilized with sodium deoxycholate.
- Lane 5 - Dialysed sample of HCMV-infected HEF membranes solubilized with Triton X-100

Bands were only visible on lanes 2 and 4 (Figure 3 on page 35) corresponding to samples treated with sodium deoxycholate, not dialysed and dialysed respectively. The lanes where samples treated with Triton X-100 were loaded had no bands whatsoever.



Figure 3. Comparison of HCMV-infected HEF cell membrane preparations using different solubilizing detergents.: Note that only lanes with sodium deoxycholate (Lanes 2 and 4) show bands.

Thus the elution experiments on the affinity chromatography columns were performed exclusively with membrane preparation samples solubilized with sodium deoxycholate since it was shown to be the most suitable detergent for solubilizing the HEF cells.

3.3 DETERMINATION OF THE PROTEIN CONCENTRATION

3.3.1 ANTIGEN CONCENTRATION

As mentioned above, the most suitable solubilizing agent found was sodium deoxycholate. Therefore, only this detergent was employed in the experiments thereafter.

The concentration of proteins in the solubilized membrane preparation was determined by means of the Bio-Rad (U.S.A) method, the estimation being performed just after the lysis of the membranes and before the samples were passed through the columns.

Using a standard protein concentration of 1.57mg/ml and a blank solution consisting of 50% sodium deoxycholate (0.4%) and 50% PBS pH 7.0, two standard curves as in Figure 6 on page 39 and Figure 7 on page 40 were obtained for determinations done in parallel with estimations for uninfected and HCMV-infected cell membrane preparations.

The membrane preparation sample was prepared for the protein determination assay and diluted to be within the range of detectable amount of protein by the standard assay (Bio-Rad). A 25% dilution of the protein sample was made.

The obtained optical density of the membrane preparations at 595nm were:

- HCMV-infected HEF membrane preparation 1.129
- Uninfected HEF membrane preparation 1.344

PROTEIN STANDARD		AVERAGE STANDARD O.D.
µg protein/mℓ	µℓ	Infected
19.84	12.7	0.264
40.03	25.5	0.610
59.97	38.2	0.772
79.91	50.9	0.955
100.00	63.7	1.112
119.94	76.4	1.219
140.04	89.2	1.283

Figure 4. Optical density values for the standard curve for protein determination used for estimation of protein content of HCMV-infected HEF cell membrane preparations.

From the standard curves shown (Figure 6 on page 39 and Figure 7 on page 40) the protein concentration of the samples were extrapolated and, bearing in mind the dilution factor, was found to be :

- HCMV-infected HEF membrane preparation 0.437 mg/mℓ of original samples

- Uninfected HEF membrane preparation 0.560 mg/mℓ of original samples

PROTEIN STANDARD		AVERAGE STANDARD O.D.
µg protein/mℓ	µℓ	Uninfected
18.84	12.7	0.303
40.03	25.5	0.545
59.97	38.2	0.669
79.91	50.9	0.979
100.00	63.7	1.048
119.94	76.4	1.206
140.04	89.2	1.254

Figure 5. Optical density values for the standard curve for protein determination used for estimation of protein content of uninfected HEF cell membrane preparations.

3.3.2 IMMUNOGLOBULIN G CONCENTRATION

The concentration of the purified IgG was standardised before binding it to the affinity chromatography column. Following the procedure of Miller and Stone (1978), PBS buffer was added to bring the optical density of the antisera to 1.4 at 280nm, when measured against PBS blank.

This concentration of IgG (1mg/mℓ) was used in the manufacture of the affinity chromatography columns.

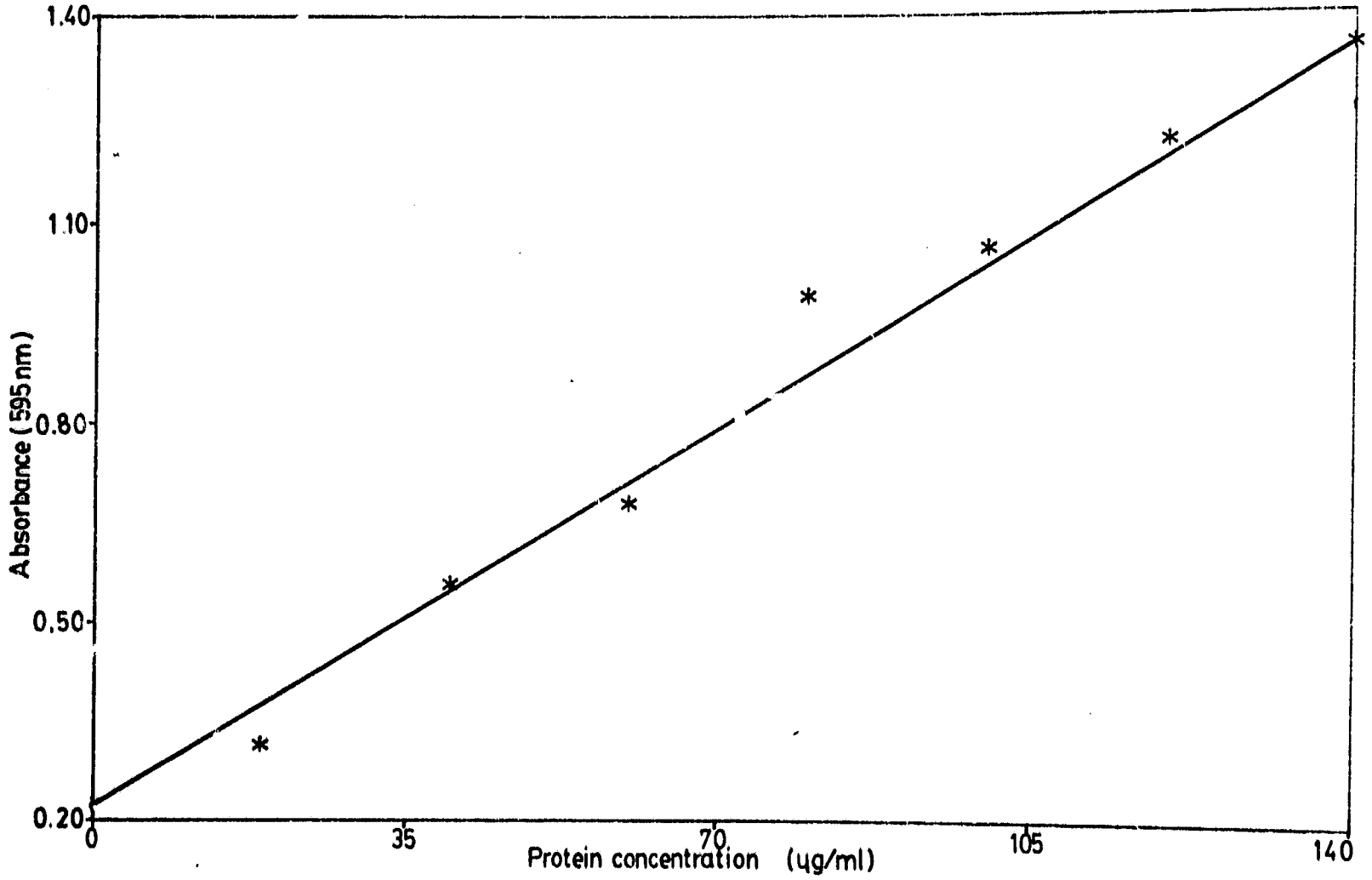


Figure 6 - Optical density values for the standard curve for protein determination of HCMV-infected HEF cell membrane preparations.

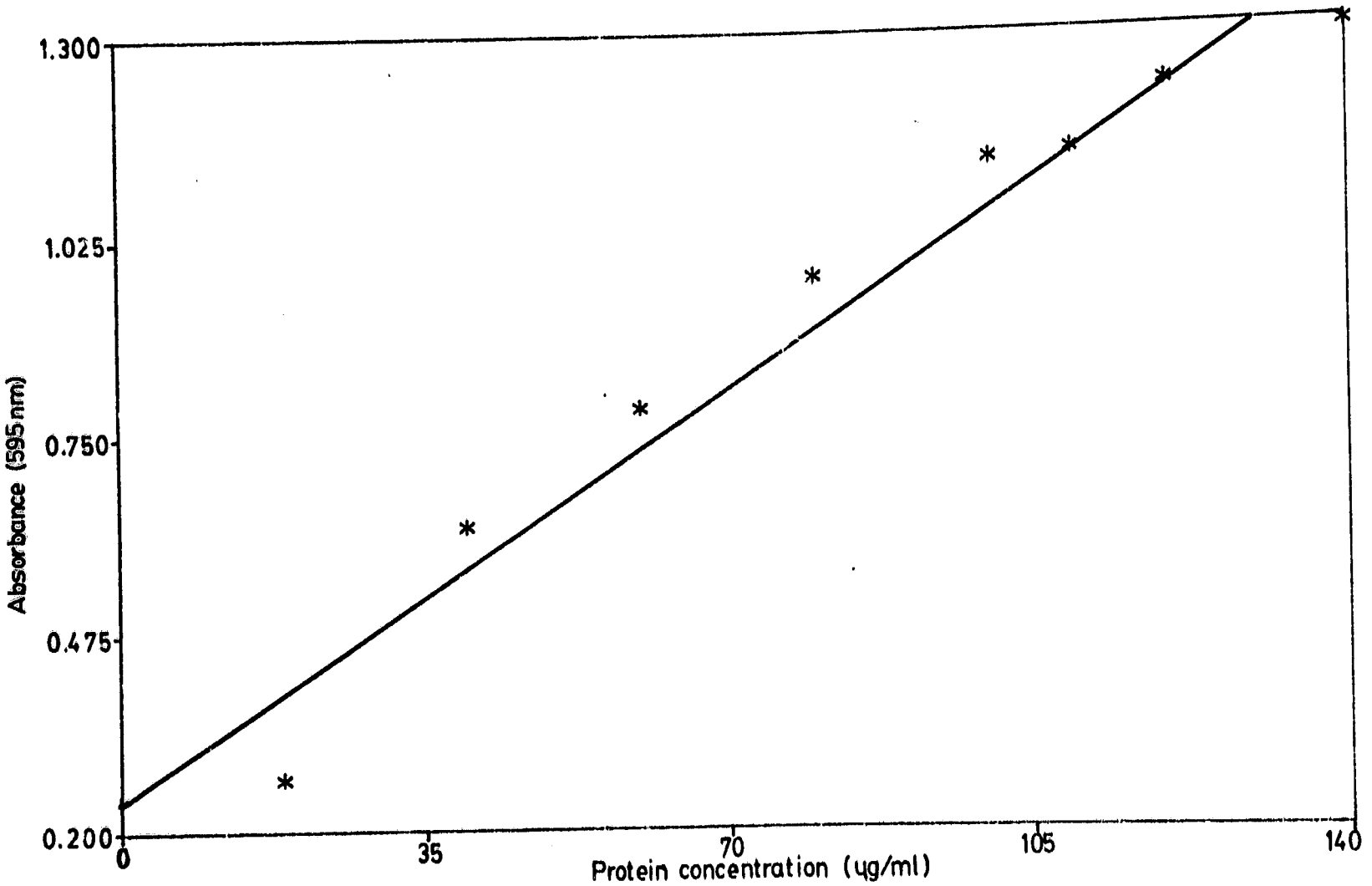


Figure 7. - Optical density values for the standard curve for protein determination of uninfected HEF cell membrane preparations.

3.4 ANTISERA PURIFICATION

IgG from the three antisera provided (against the glycoproteins 62Kd, 54Kd and 90Kd) were obtained by passage through a Protein A-Sepharose (Pharmacia Fine chemicals, Uppsala, Sweden) affinity chromatography column. (Figure 8 on page 42)

The concentration and amount of IgG obtained from each antiserum were as follows :

	54Kd IgG	62Kd IgG	90Kd IgG
Protein concentration	1mg/mℓ	1mg/mℓ	1mg/mℓ
Protein amount	3mg	3.54 mg	1.6 mg

This amount of IgG determined the value of each of the affinity chromatography columns to be used thereafter, the ratio being within the range suggested by Pharmacia fine chemicals (Uppsala, Sweden).

	54Kd IgG	62Kd IgG	90Kd IgG
Volume of Column	3mℓ gel	2.5mℓ gel	2mℓ gel

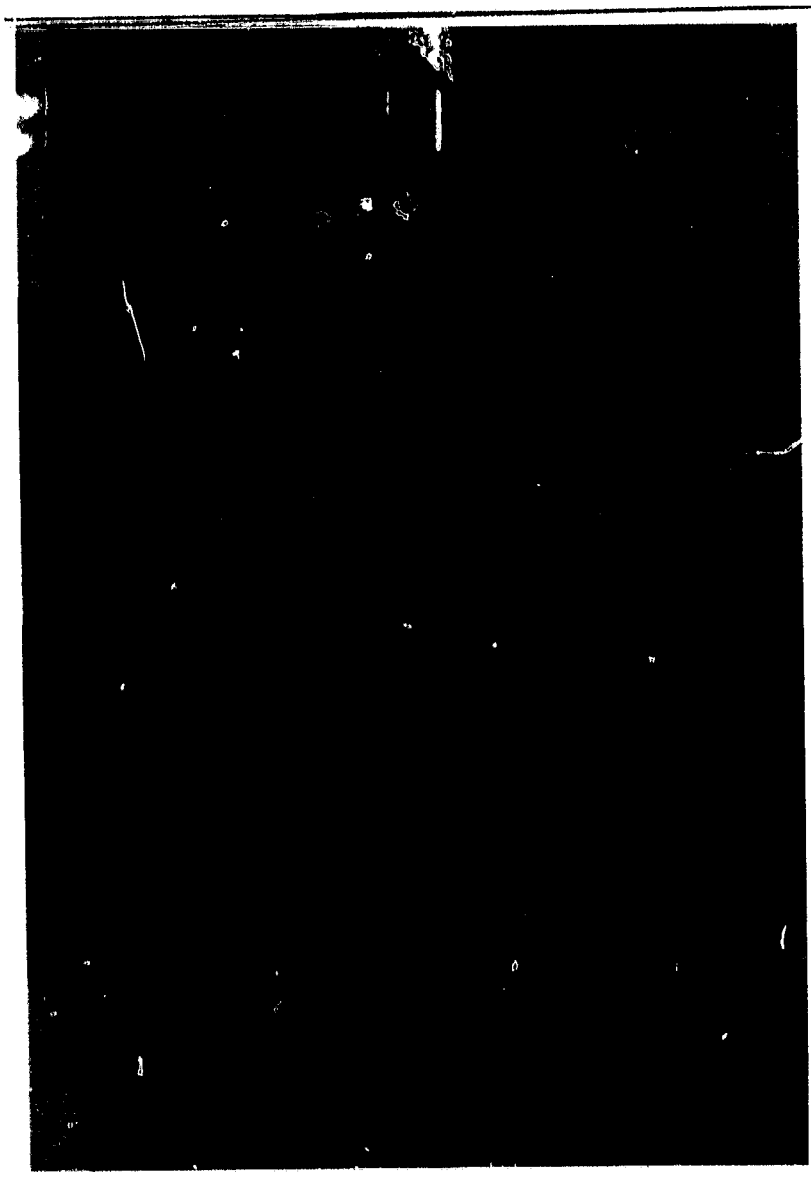


Figure 3. Protein A-Sepharose affinity chromatography column for IgG purification

3.5 ANTIGEN PURIFICATION

The affinity chromatography columns (Figure 9 on page 44) were prepared as follows :

- **54Kd column :**

  1 mg IgG per mℓ of gel with a final volume of 3mℓ of gel and 3mg of IgG.

- **62Kd column :**

  1.43mg IgG per mℓ of gel with a final volume of 2.5mℓ of gel and 3.57mg IgG.

- **90Kd column :**

  0.8mg IgG per mℓ of gel with a final volume of 2mℓ of gel and 1.6mg IgG.

Since the best results were obtained using sodium deoxycholate instead of Triton X-100 as detergent to solubilize the membranes, the sodium deoxycholate was used thereafter in all experiments.

Different elution buffers were utilised to elute the polypeptides from the bound antibodies on the column.

Three experiments were performed on each of the three columns as follows :

1. 400 µℓ of uninfected HEF membranes (Total protein concentration of 0.224 mg) were passed through the column. The high pH (11.5) elution buffer was used during the elution procedure.

2. 500 µℓ of HCMV-infected HEF membranes (Total protein concentration of 0.218mg) were passed through the column. The high pH (11.5) elution buffer was used during the elution procedure.

3. 500 µℓ of HCMV-infected HEF membranes (Total protein concentration of 0.218mg) were passed through the column. The low pH (4) elution buffer was used during the elution procedure.

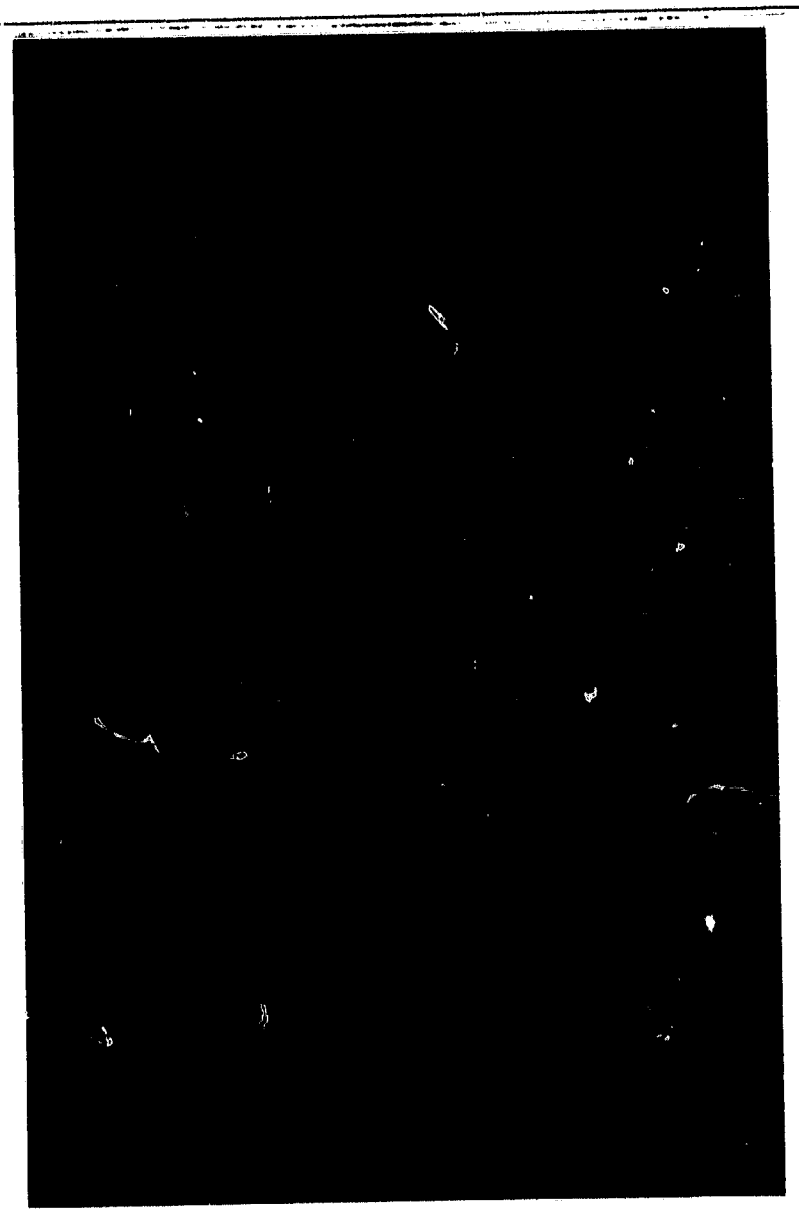


Figure 2. Cyanogen-Bromide activated sepharose affinity chromatography column for antigen purificat

Because of the different amount of IgG coupled to each column, the potential membrane protein/IgG ratio for each column and for each experiment

was different, although there was little variability between the experiments with membranes from infected and uninfected cells.

	54Kd	62Kd	90Kd
Uninfected HEF membrane/IgG	0.672	0.799	0.358
Infected HEF membrane/IgG	0.654	0.778	0.348
Uninfected HEF membrane/Volume gel	0.672	0.560	0.448
Infected HEF membrane/Volume gel	0.654	0.545	0.436

The eluting fractions were collected in eppendorf tubes at a rate of 15 seconds per drop, and the optical density at 280nm measured on the spectrophotometer against the elution buffer as blank.

Because the coupling buffer and the elution buffer have different absorbances at 280nm, it was not possible to determine the amount of protein of each fraction. Thus, the fractions were collected until the optical density at 280nm was near zero. The resulting elution fractions presented a volume of 7.5mℓ which were subsequently dialysed overnight and concentrated as mentioned in "2.0 Materials and Methods" on page 20. The concentration step is performed to load all the eluted protein in the same well of the PAGE gel.

3.6 POLYACRYLAMIDE GEL ELECTROPHORESIS

The fractions resulting from the eluted columns were, once concentrated, run on gels. All the results from the different experiments performed on

each column were run on the same gel to allow comparisons. Each gel was run in duplicate, one was stained to visualise results of the protein separation and the other one was used for blotting purposes.

The gel prepared to detect proteins eluted from the column with antiserum to the 90Kd glycoprotein didn't show any bands in any of the three lanes other than the marker. The gels of proteins eluted from the 54Kd and 62Kd glycoprotein antibodies didn't show any bands on lanes 3 and 4, which are the lanes for the HCMV infected HEF membranes and the uninfected HEF membranes eluted at pH 4 and pH 11.5 respectively. However, a faint band appeared on lane 2 of both gels corresponding to the HCMV infected HEF membranes eluted at pH 11.5 (Figure 10 on page 47 and Figure 11 on page 48)

The molecular weight estimation of the band appearing on lane 2 of the gels from proteins eluted from 54Kd and 62Kd antibody columns was done by extrapolation with the molecular weight markers. (Figure 12 on page 49 and Figure 13 on page 50). The molecular weight of the protein of the bands were estimated to be between 54 and 56Kd and between 60 and 62Kd respectively.



Figure 10. SDS-PAGE prepared to detect proteins eluted from the column with antiserum to the 54Kd glycoprotein:
Lane 1 - Marker
Lane 2 - HCMV-infected HEF membranes eluted at pH 11.5
Lane 3 - HCMV-infected HEF membranes eluted at pH 4.0
Lane 4 - Uninfected HEF membranes eluted at pH 11.5
Note that a faint band is visible on lane 2. The molecular weight was found to be between 54-56Kd.

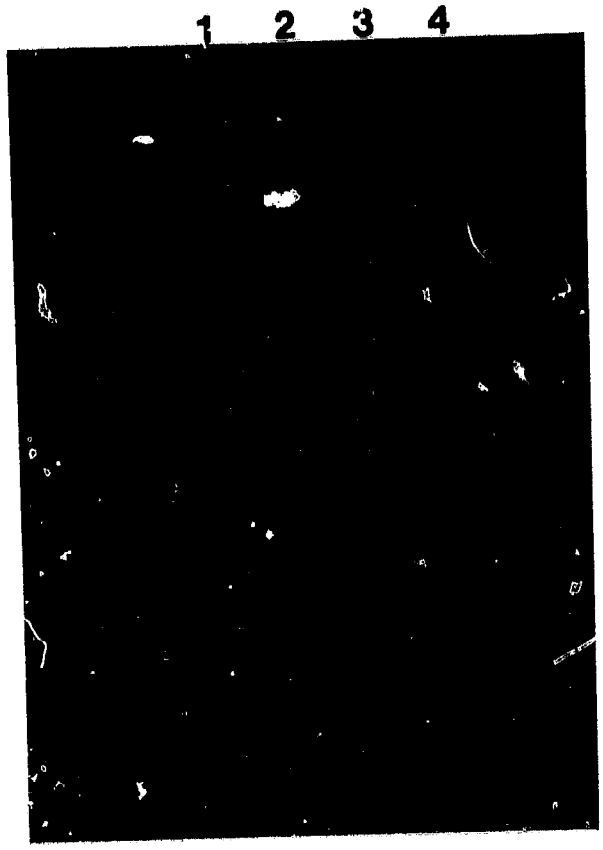


Figure 11. SDS-PAGE prepared to detect proteins eluted from the column with antiserum to the 62Kd glycoprotein:
Lane 1 - Marker
Lane 2 - HCMV-infected HEF membranes eluted at pH 11.5
Lane 3 - HCMV-infected HEF membranes eluted at pH 4.0
Lane 4 - Uninfected HEF membranes eluted at pH 11.5
Note the faint band appearing on lane 2.
The molecular weight of the protein was found to be between 62-64Kd.

3.7 PROTEIN BLOTTING

The proteins separated through polyacrylamide electrophoresis were tested against the antisera.

The result of the protein blotting was negative in all three cases. Neither of the antisera could react against any protein on the gels.

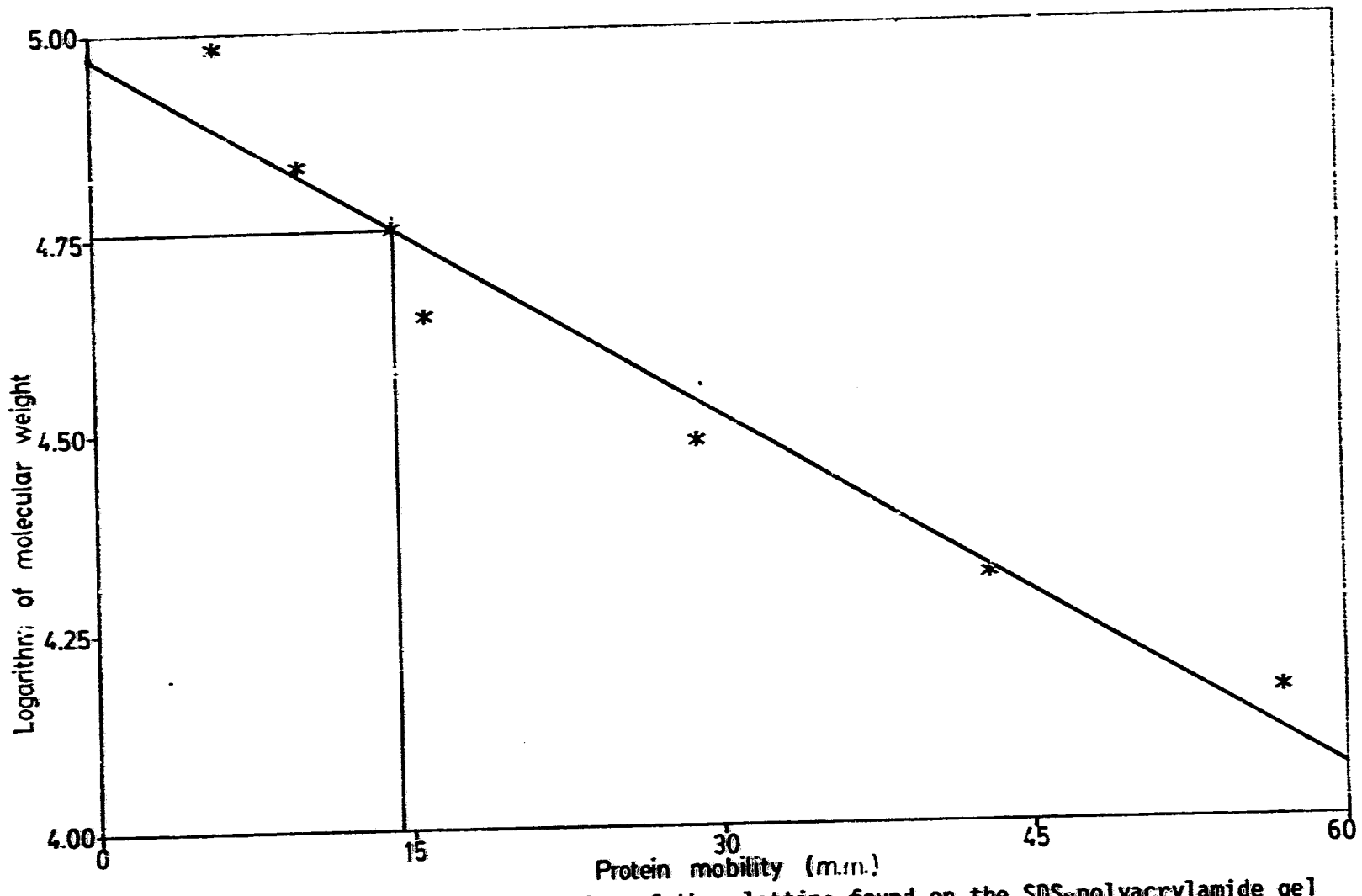


Figure 12 - Molecular weigh estimation of the plotting found on the SDS-polyacrylamide gel prepared to detect proteins eluted from the column with antiserum to the 54kd glycoprotein.

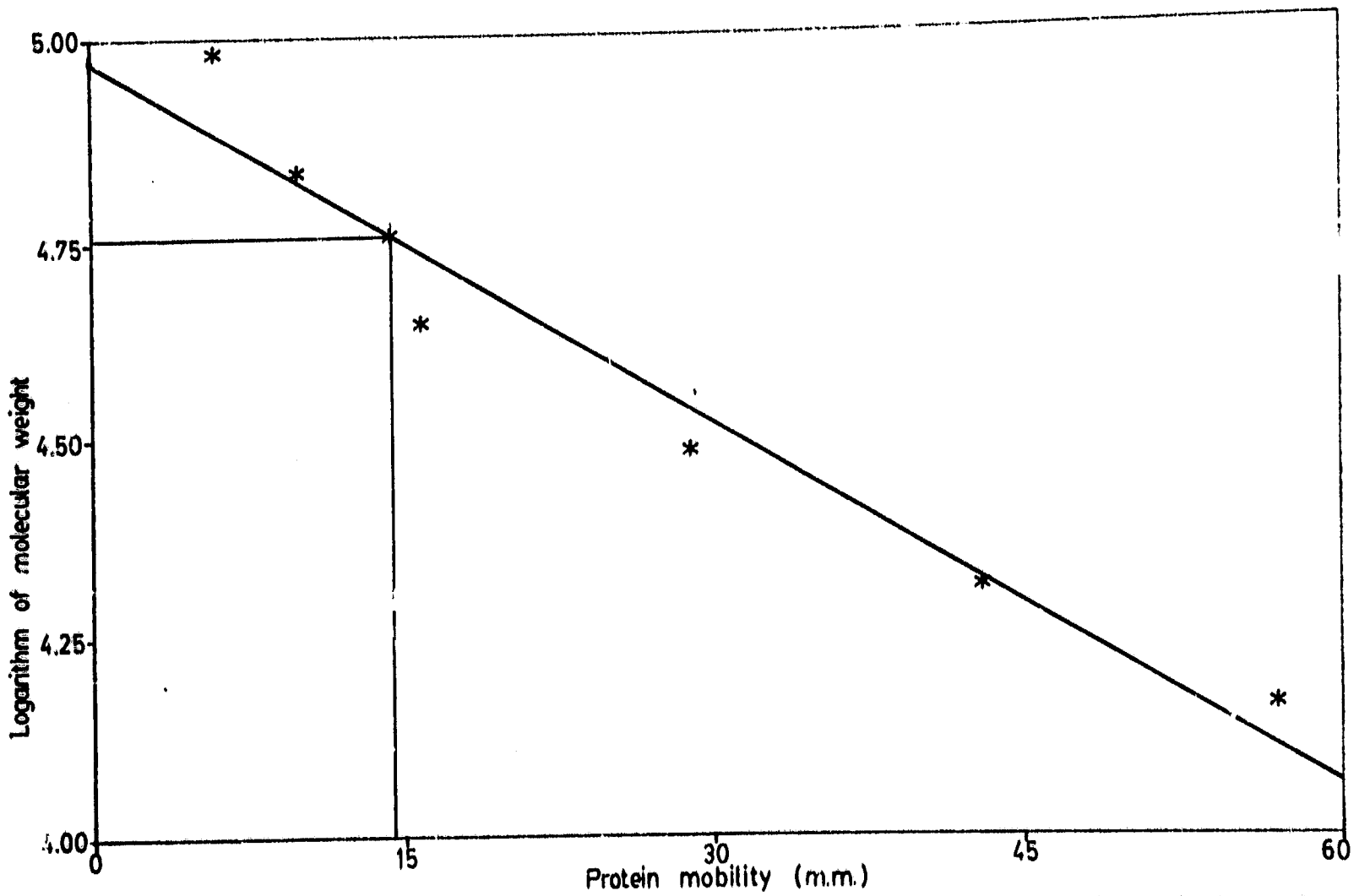


Figure 12 - Molecular weigh estimation of the plotting found on the SDS-polyacrylamide gel prepared to detect proteins eluted from the column with antiserum to the 54kd glycoprotein.

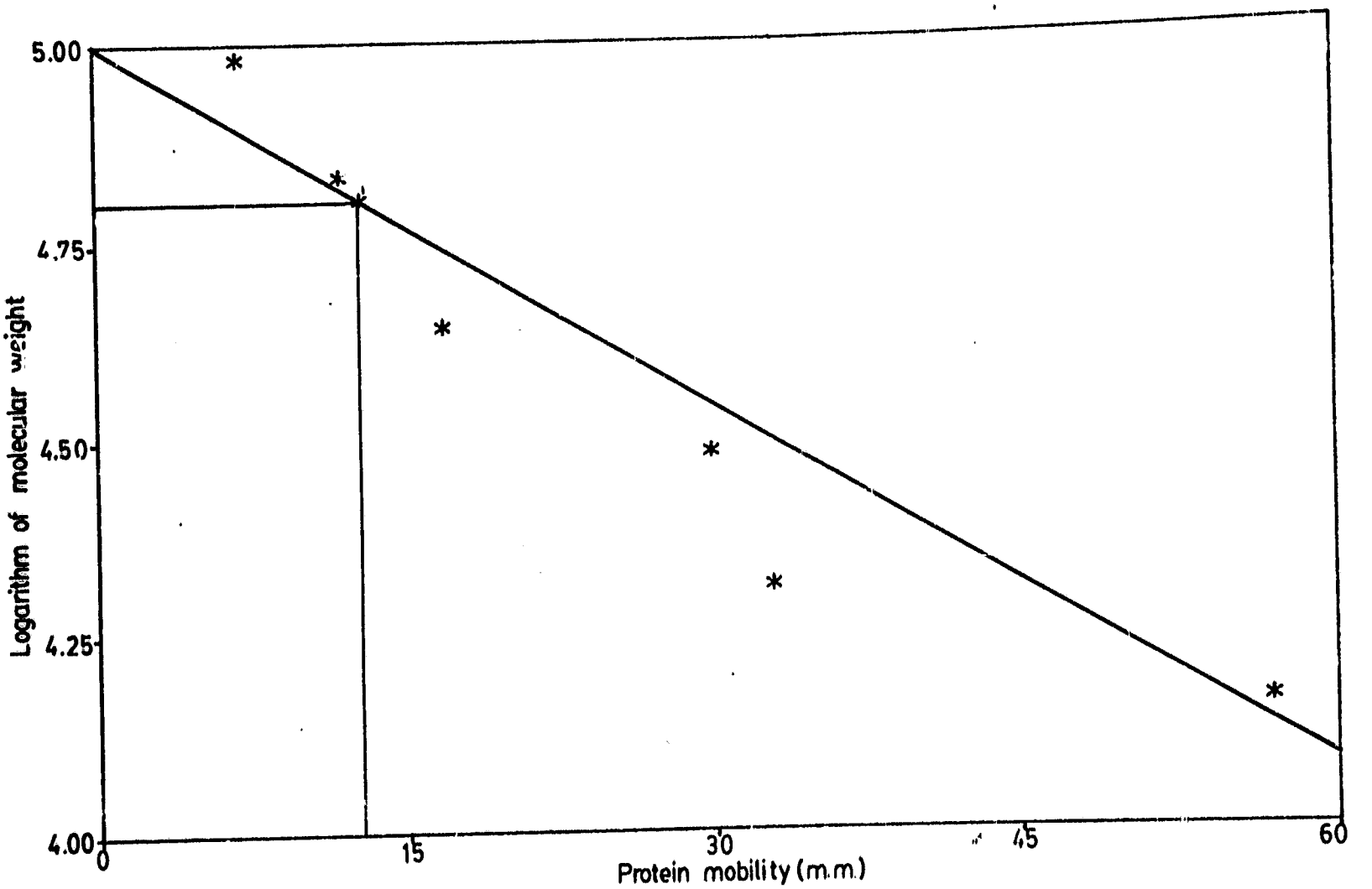


Figure 13 - Molecular weigh estimation of the protein found on the SDS-polyacrylamide gel prepared to detect proteins eluted from the column with antiserum to the 62kd glycoprotein.

4.0 DISCUSSION

The growth of uninfected HEF cells was deeply reduced after the 25^{th}-30^{th} passage of the same cell line. Some apparent mutations were observed to occur and the characteristic morphology of the cells no longer was seen. These changes in morphology were present together with changes in the medium colour, presumably due to the above mentioned mutations. These cells were discarded for any experimental work.

Earlier passages grew well presenting typical morphology. The growth rate was high and the 90% confluency of the cells was reached within four days after trypsinization took place.

Virus stock obtained had high titer (10^9 PFU/mℓ) although it was subsequently diluted to 19^7 PFU/mℓ. Within five days of infection the cells were ready to be harvested and used in experimental work.

Although a number of methods to lyse cells have been reported in the literature, the most convenient technique is to be determined for each particular case. Furthermore, using different techniques on HCMV-infected HEF cells different proteins are reported to be recovered from its membrane (Schramm, 1984). From the experience of workers at this same laboratory on 54Kd, 62Kd and 90Kd glycoproteins, the lysis method from Krah and Crawell (1982) was selected to isolate the membranes from both HCMV-infected and uninfected cells. The results suggest that the method works successfully for this purpose, and only a few adjustments in buffer concentration had to be introduced. The 0.2M phosphate buffer showed the best membrane disruption and the most homogeneous membrane preparation under a light microscope. The FMA method was initially discarded for this work albeit its good results in some cases (Sullivan-Tailyour, 1984) since

the fluorescein mercuric acid interferes with the affinity chromatography column.

The selection of a suitable detergent is critical for the proper membrane solubilization. From the theory of detergents, either a bile salt or a nonionic detergent could be a suitable detergent for cell membrane solubilization. However, from the experience in this laboratory (personal communication with R. Saunders) Chaps and Octyl β-D Glucopyranoside detergents, both nonionic detergents, were not found to be suitable whereas sodium deoxycholate gave positive results. Thus, sodium deoxycholate and Triton X-100 were chosen among these two detergent categories. The selection of the former became clear when no bands could be observed on PAGE gels after running membrane samples treated with Triton X-100, whereas several bands were seen on lanes corresponding to membrane samples solubilized with sodium deoxycholate. (Figure 3 on page 35)

The concentration of Triton X-100 used was limited by the Bio-Rad method itself since a higher Triton X-100 concentration interferes with the method, thus, becoming unreliable. The concentration of sodium deoxycholate was the same successfully used before (Personal communication with R. Saunders)

Since temperature is a critical condition for the effectiveness of bile salts as solubilizing agent (Helenius *et al*, 1983), the whole lysis procedure was performed at 4°C as reported in the literature. (Krah and Crawell, 1982).

Two different purification techniques were considered for obtaining the IgG from the antisera. The rivanol method (Walker *et al*, 1971) appears to be long and less successful. While, the purification of IgG from antisera through an affinity chromatography column is faster and gives better IgG quality (Personal communication with M. Sibara). Protein A

Sepharose CL-4B was used to bind the IgG to the affinity chromatography column. The results from the purification procedure appears to be satisfactory when the final IgG concentration values are considered.

The antigen was purified using a cyanogen bromide activated sepharose 4B (Pharmacia, Uppsala, Sweden) affinity chromatography column. The use of such a technique to purify virus glycoproteins is not new. Wroblewska *et al* (1985), report the use of CNBr-activated sepharose 4B column to purify Varicella-Zoster virus glycoprotein gp1/gp3. The affinity purified glycoproteins were used as an immunogen to stimulate the production of monospecific neutralizing antibodies in rabbits. Eisenberg *et al* (1982) reported that glycoproteins gD-1 and gD-2 of Herpes Simplex virus types 1 and 2, respectively, were purified on an immunoadsorbent column consisting of the type-common monoclonal antibody HD-1 linked to sepharose. Each glycoprotein was of sufficient purity, quantity and biological activity to be used for immunological and biochemical studies and each glycoprotein induced high titers of type-common monospecific neutralizing antibody in mice.

An alternative to the above procedure is to use an end-over-end rotator instead of a column. Rasmussen *et al* (1985), used CNBr-activated sepharose beads to isolate by immunoaffinity chromatography three polypeptides of HCMV believed to be targets for virus neutralizing antibodies. The study showed that the purified polypeptides are immunogenic in guinea pigs for CMV-neutralizing antibodies. For the present study, the column method was utilised.

The amount of antibody bound to the column varies in different reports. Pharmacia fine chemicals recommends the use of 5-10mg protein/ mℓ of gel. Wroblewska *et al* (1985) used 6mg of purified IgG per mℓ of gel for the Varicella-Zoster virus glycoproteins and Eisenberg *et al* (1982), used 3 mg IgG per mℓ of gel for the Herpes Simplex virus glycoprotein. Rasmussen

et al (1985) in their work on human cytomegalovirus used a different IgG concentration for every polypeptide. They used 2mg of IgG per mℓ of gel for the p86 and 10mg of IgG per mℓ of gel for the other two polypeptides p130 and p55.

The amount of purified IgG per mℓ of gel in this work was 0.8mg for the 90Kd column, 1mg for the 54Kd column and 1.43mg for the 62Kd column. These concentrations being in the range of the minimum binding capacity found in the literature (Goding, 1980). This low concentration was used due to the fact that the amount of antisera available was limited.

The column volume utilised by other workers are as high as 7mℓ (Eisenberg, 1982). Once again, the limited amount of sera made a bigger column impossible. The volume used, 2mℓ for the 90Kd column, 3mℓ for the 54Kd column and 2.5mℓ for the 62Kd column, may be slightly small, although this point is unlikely to be critical as the columns can be re-utilised many times to obtain sufficient quantity.

The initial idea was to monitor the protein concentration from the elution fraction via measuring the absorbance at 280nm. Thus, the elution procedure could have been ended when no more proteins appeared in the sample. An automatic fractionator connected to a peristaltic pump, was used for that purpose. However, the problem encountered was that the elution buffer has a different absorbance at 280nm than the coupling buffer. It was impossible to set the absorbance baseline since the whole elution procedure was an absorbance gradient between both buffers. The problem was overcome by eluting the column until the absorbance of the fraction using the eluting buffer as a blank was near zero and, hence, only pure buffer was being eluted. The total fraction was then concentrated as explained in "2.0 Materials and Methods" on page 20Wroblewska *et al* (1985), used Varicella Zoster virus infected cell lysates from 10^8 cells from the column, whereas only 5×10^7 cells were used in this work. This fact to-

gether with the low IgG : gel ratio and the small column sizes could help to explain why only low amounts of purified proteins were obtained as seen by the SDS-PAGE gels. Nevertheless sufficient material was obtained to ascertain the effectiveness of the different buffers.

The elution buffer appears to have a critical effect on the elution procedure since only one of the two buffers used eluted sufficient protein to be visible on the gels, this being the buffer of pH 11.5. The low pH (4) buffer had a lower effectiveness for elution, no bands being visible on gels loaded with concentrated elution.

The results of the SDS-PAGE gels indicate that only a small amount of the antigen was present in the elution fraction run on gels. However it must be realised that in studies to identify the novel CMV induced protein in HEF cells utilised 10^7 cells to produce sufficient membrane protein for visualisation on gels (Sullivan-Tailyour, 1984). The band visible on the PAGE analysis from the eluted column coupled with antibodies against the 62Kd protein are clearer than those from the eluted column coupled with antibodies against the 54Kd proteins. It is interesting to remark here that the column coupled with antibodies against the 62 Kd protein had the highest IgG concentration, 1.43mg/mℓ, followed by the column coupled with antibodies against the 54Kd protein with 1mg/mℓ The column coupled with antibodies against the 90Kd proteins with a low 0.8mg IgG per mℓ of gel did not show any bands on the gel. An increase in the concentration of antibody may result in a stronger band on each gel.

Only one band can be seen on the PAGE analysis of the eluted columns coupled with antibodies against the 54Kd and 62Kd proteins, suggesting that the IgG used are highly monospecific for each glycoprotein. Obviously this suggestion can not apply for the column coupled with antibodies against the 90Kd protein, since no bands whatsoever could be seen. However, since only low amounts of protein were available for gel

electrophoresis, and the samples were too small to allow protein concentration to be ascertained before PAGE, more experiments should be performed before relying on the present sera for purification purposes. With a higher loading of proteins on the gels other bands might also appear, disqualifying the present sera for future work.

The results of the protein blotting were negative with no reaction between the proteins on the gel and the corresponding serum being observed. However, as the bands were very faint, insufficient protein may have transferred to the nitrocellulose paper. The concentration of IgG used in the blotting procedure was appropriate, the same blotting procedure having been used successfully for other experiments.

In conclusion the current experiments have determined conditions for cell rupture and membrane solubilisation which yield proteins capable of being separated by PAGE. The solubilised 54Kd and 62Kd proteins appear to be selectively retained by sepharose columns coupled to antibody raised against denaturated protein eluted from the PAGE slices and a high pH buffer is suitable for their elution.

Further studies will need to be performed to substantiate these results. These include the use of higher amounts of membrane preparation per column and a higher antibody to gel ratio. If indeed the sera are monospecific, they will enable the purification of these CMV-induced proteins in sufficient quantities for structural studies on the proteins and so facilitate the future development of a CMV subunit vaccine.

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APPENDIX 1

CELL CULTURE

REAGENTS AND SOLUTIONS

1. 1 - PHOSPHATE BUFFERED SALINE (PBS), PH 7.0

(Calcium and Magnesium free)

Component	Quantities for 1 litre
NaCl	8.000g
KCl	0.200g
KH_2PO_4	0.200g
$Na_2HPO_4.12H_2O$	2.890g

Each salt was dissolved in glass distilled water in the order listed. The solution was autoclaved for 20-30 minutes, and dispensed into 200mℓ sterile bottles. From each bottle dispensed, 1-5mℓ was cultured in thioglycollate broth to test the sterility. The solution was stored at 4°C

2. TRYPSIN-ETHYLENEDIAMINE TETRAACETIC ACID (EDTA) SOLUTION

- 0.25% trypsin (w/v)
- 0.05% EDTA (w/v)

In calcium and magnesium free PBS

At a pH 7.0-7.5

3. THIOGLYCOLLATE BROTH FOR BACTERIOLOGICAL STERILITY TEST

24g Thioglycolate (Difco) powder was added to 500mℓ glass distilled water and mixed. To this 5g dextrose was added. Sufficient distilled water was then added to mak· 1ℓ. The broth was dispensed in 5mℓ amounts and autoclaved for 20-30 minutes and stored at room temperature in the dark.

APPENDIX 2

MEMBRANE ISOLATION AND SOLUBILIZATION

1. PREPARATION OF THE LYSIS BUFFER

a. 0.2M PHOSPHATE BUFFER PH 7.0

To 200mℓ glass distilled water 5.44g KH_2PO_4 was added.

To 200mℓ glass distilled water 7.00g K_2HPO_4 was added.

The above solution were mixed and the pH checked to ensure that it was 7.0

2. DETERGENT SOLUTION

a. TRITON X-100

50% PBS

50% of 0.1% Triton X-100

b. SODIUM DEOXYCHOLATE (D.O.C)

50% PBS

50% of 0.4% D.O.C

APPENDIX 3

ANTISERUM PURIFICATION-PROTEIN A-SEPHAROSE AFFINITY CHROMATOGRAPHY

1. REAGENTS

a. BUFFER A :

- 0.016M boric acid
- 0.012M NaCl
- 0.0025M NaOH
- 0.1mM phenylmethylsulphonyl fluoride (PMSF)
- 0.02% NaN_3

Buffer pH should be 8.0

b. BUFFER B :

- 0.1M glycine-HCl
- 0.1mM PMSF
- 0.02% NaN_3

Buffer pH should be 3.0

2. PROTEIN A COLUMN

10mℓ slurry of protein A sepharose CL-4B packed in K9/15 column equilibrate with buffer A.

APPENDIX 4

ANTIGEN PURIFICATION-CYANOGEN BROMIDE ACTIVATED-SEPHAROSE AFFINITY CHROMATOGRAPHY

REAGENTS :

1. COUPLING BUFFER :

- 0.2M $NaHCO_3$
- 0.5M NaCl

Buffer pH should be 8.5

2. ACETATE BUFFER :

STOCK SOLUTIONS :

A : 0.2M solution of acetic acid (11.55mℓ in 1000mℓ)

B : 0.2M solution of sodium acetate (16.4g of $C_2H_3O_2Na$ or 27.2g of $C_2H_3O_2Na.3H_2O$ in 1000mℓ)

41.0 mℓ of A + 9.0mℓ of B, diluted to a total of 100mℓ

Buffer pH should be 4.0

3. ELUTION BUFFERS :

Volume 2, ed. J Craig Venter, Len C. Harrison, Alan R. Liss, Inc., New York.pp 1-13.

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a. LOW PH ELUTION BUFFER :

Acetate buffer, as above

b. HIGH PH ELUTION BUFFER :

50%(v/v) ethylene glycol in 0.1% Triton X-100

Buffer pH should be 11.5

APPENDIX 5

PROTEIN GEL ELECTROPHORESIS

1. REAGENTS

a. 1M TRIS-HCL (PH 8.8)

60.6g tris

7.3 mℓ 11.6N HCl

Added to 500mℓ glass distilled water

b. BATH BUFFER (10× CONCENTRATED)

30.3g tris

144.1g glycine

10.0g SDS

Added to 1ℓ glass distilled water

c. ACRYLAMIDE STOCK SOLUTION

150.0g Acrylamide

4.0g bis-acrylamide

pump, to circulate the buffer, the water cooling system and a current of 40mA per gel was switched on.

3. SAMPLE PREPARATION

a. MOLECULAR WEIGHT MARKERS

Low molecular weight electrophoresis markers (Pharmacia, Uppsala, Sweden) containing the following components were used :

Component	Molecular weight (Daltons)
Phosphorylase b	94,000
bovine serum albumin	67,000
Ovalbumin	43,000
Carbonic anhydrase	30,000
Soybean trypsin inhibitor	20,100
α-Lactalbumin	14,400

b. SPLITTING SOLUTION

- 2% SDS
- 5% 2-mercaptoethanol
- 5M Urea

Membrane samples were solubilized in a ratio of 1:1 with the above solution.

For the molecular weight markers, 100µℓ of a splitting solution was added per vial of powder, but of this only 10µℓ was applied to the gel.

All samples were heated in a boiling water bath for 5 minutes prior to laying onto the gel.

4. FI[illegible]E

15% TCA in distilled water

5. STAINING OF GELS

- 0.25% coomassie brilliant blue
- 45.00% methanol
- 7.00% glacial acetic acid

Gels were placed in the stain solution and left overnight on a shaker at low speed. The container was covered to minimise evaporation.

6. DESTAINING OF GELS :

- 45.00% methanol
- 7.00% glacial acetic acid

Made up to 3ℓ with distilled water

Gels were destained for 1-2 hours.

APPENDIX 6

PROTEIN BLOTTING

REAGENTS :

1. ELECTRODE BUFFER

- 25 mM Tris
- 192 mM glycine
- 20% methanol (v/v)

Buffer pH should be 8.3

2. WASHING BUFFER

- 10mM tris-HCl

Buffer pH should be 7.4

Added to 500mℓ glass distilled water

d. 10% SDS made up in 100mℓ glass distilled water

e. 1.5% ammonium persulphate freshly prepared each time

f. TEMED

g. Glass distilled water

2. GEL PREPARATION

VOLUMES OF REAGENTS USED :

Reagent	Volume for 12% gel
Acrylamide stock solution	48.4 mℓ
1M tris-HCl, pH 8.8	45.0 mℓ
glass distilled water	19.6 mℓ
10% SDS	1.2 mℓ

The above were mixed and degassed for 10 minutes and then the following were rapidly added :

- TEMED 30µℓ
- 1.5% ammonium persulphate 6mℓ

The above components were mixed well and poured into gel holders and left for 1 to 2 hours to polymerise. Glass distilled water was layered on top to prevent dehydration. Once set, the gel cassettes were placed in an electrophoresis vessel containing three litres bath buffer. The

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