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Human cytomegalovirus induced plasma membrane glycoproteins in fibroblasts

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HUMAN CYTOMEGALOVIRUS INDUCED PLASMA MEMBRANE GLYCOPROTEINS IN FIBROBLASTS.

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from

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by

Shan Holmes, BSc (Hons), MSc (Witwatersrand).

DECLARATION.

I declare that this thesis is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy in the University of Wollongong, Wollongong. It has not been submitted before for any degree or examination in any other University.

(SHAN HOLMES)

THIS 26TH day of JANUARY, 1990.

I would like to thank my supervisor Prof. H.M Garnett, for her advice and encouragement.

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The Second International Cytomegalovirus Workshop, 1989, San Diego, USA.

LIST OF ABBREVIATIONS.

BSA	Bovine serum albumin
CBB	Coomassie brilliant blue
CPE	Cytopathic effects
DAB	3, 3' diaminobenzidine tetrahydochhloride
DMSO	Dimethylsulfoxide
EBV	Epstein-Barr virus
EDTA	Ethylenediamine-Tetraacetic-acid
ELON	p-methylaminophenol sulphate
FCS	Foetal calf serum
FMA	Fluorescein mercuric acetate
h	hour(s)
hr.	hours.
HBSS	Hanks Balanced Salt Solution.
HCMV	Human cytomegalovirus
HEF	Human embryonic fibroblasts
HFF	Human foreskin fibroblasts
HMW	High molecular weight
HMWC	High molecular weight complex
HSV	Herpes simplex virus
IE	Immediate early
IgG	Immunoglobulin G
kDa	kilodaltons
KM	Potassium magnesium
Mab	Monoclonal antibody
MCMV	Murine cytomegalovirus
MEM	Minimal essential medium
m.o.i.	Multiplicity of infection
NK	Natural killer

PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PFU	Plague forming unit
PPO	2, 5 -diphenyloxazole
rpm	revolutions per minute
SDS	Sodium dodecyl sulphate
TBS	Tris bufferd saline
TEMED	Tetramethylethylenediamine
TN	Tris sodium chloride
Tris	Trihydroxyaminomethane
v/v	Volume per volume
VZV	Varicella Zoster virus
w/v	Weight per volume

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ABSTRACT.

The relationship of novel HCMV induced plasma membrane glycoproteins of M.W. 54, 62 and 94 kDa to proteins of the virion envelope and normal cell plasma membranes was investigated. Plasma membrane preparations were isolated from metabolically labelled HCMV infected and uninfected fibroblasts by the FMA and two phase polymer systems procedures, and analysed by SDS PAGE, fluorography and image subtraction analysis, for the initial characterization of HCMV induced proteins of M.W. 26,32-34,38,48,54-55,62,68,72-74,86,92-94,105,130 and 150 kDa.

The reactivity of antisera raised against virus induced plasma membrane proteins of 54, 62 and 94 kDa with ^{125}I labelled virions and detergent extracted and osmotically fragile virion envelope proteins was assessed by immunoprecipitaion analysis, reducing SDS PAGE and fluorography. All three antisera were reactive with at least one protein of M.W. 54-58 kDa and anti- 54 kDa and anti- 62 kDa sera, were shown to be specfic by immunoaffinity absorption chromatography for reduced HCMV induced novel plasma membrane proteins of 54 and 62 kDa respectively. Both these antisera also neutralized virus *in vitro* in the absense of complement. All three antisera were also shown to react with the nuclear and plasma membranes of infected fibroblasts by indirect immunoperoxidase immunobinding assays.

Rabbit antisera raised against HCMV induced plasma membrane proteins of 54, 62 and 94 kDa, guinea pig monospecific polyclonal antisera reactive with the gB virion envelope proteins 55/130 kDa, and monoclonal antibodies specific for the virion gB complex, 15D8 (Rasmussen *et al.*, 1985), CH 28-2 (Pereira *et al.*, 1984), 7-17 (Britt *et al.*, 1985) and α 52 (Farrar and Greenaway, 1986) were used to assess the antigenic association of virion envelope proteins and HCMV induced and normal plasma membrane proteins.

Immunoblot and immunoprecipitation analyses of HCMV induced plasma membrane proteins with anti- 54, 62 and 94 kDa sera indicated that HCMV induced plasma membrane proteins of 54, 62 and 94 kDa were antigenically related and reacted with HMWC'S of 130-150 and 500kDa in plasma membrane preparations from infected fibroblasts. Anti-54 kDa sera was shown to be virus specific whereas anti-62 and anti-94 kDa sera demonstrated a low affinity for host cell proteins although a HMWC of 140 kDa could possibly be virus specific.

HMWC's of 130-150 kDa, also reported for the virion gB glycoprotein complex, were detected when HCMV induced plasma membrane proteins were immunoprecipitated with monospecific polyclonal sera 4PP and 6PP and Mab 15D8 and analysed under non reducing gel conditions. Serum 4PP and Mab 15D8 were highly virus specific whereas serum 6PP cross reacted with low affinity to host cell proteins. Plasma membrane preparations of HCMV infected and uninfected fibroblasts immunnoprecipitat ed with Mabs 15D8, 7-17 , CH28-2 and analysed by reducing SDS PAGE and fluorography showed that proteins of similar M.W. to proteins of the gB glycoprotein complex of the virion envelope, namely 130, 94, 68 and 55 kDa were also present in the plasma membranes of infected cells. However HMWC's of 130-150 kDa and reduced proteins in the molecular weight region of 130, 68-70, 55 and 28 kDa were also detected in normal cell plasma membrane proteins and in plasma membrane preparations isolated at 12h and 48h p.i. with Mab 15D8 when the autoradiographs were exposed for an extended time, indicating that normal cell proteins cross react with virion envelope proteins that neutralize virus infectivity *in vitro*.

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INTRODUCTION

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1. INTRODUCTION.

1.1. Human Cytomegalovirus.

Cytomegaloviruses belong to a group of DNA containing viruses, the herpesviridae. Although they are structurally and biologically similar to most viruses belonging to this family, several characteristics are considered to be unique to cytomegaloviruses, the major features being:

1.1.1. Physical properties.

Human cytomegalovirus (HCMV) has an icosahedral capsid configuration with 162 capsomeres and contains a double-stranded linear DNA genome of approximately 230 kilo-bases. The size of the mature enveloped virion particle ranges from 180μm-250μm. The genome contained within the electron dense core consists of a long-unique (L) and short-unique (S) region, marked by different repeat sequences that are inverted relative to each other (Stinski *et al.*, 1983). As such, four isomeric arrangements of the HCMV genome occur. The unique sequences make up the majority of the viral genome, although eleven percent (%) the genome is repeated. The genome is also 50% larger than the HSV1 genome and is considered to have a 30% greater molecular complexity. Many of strains of HCMV have been isolated and those commonly used in the laboratory include the Davis, Towne, AD 169, Esp, 751 and Kerr strains. It has been shown that about an eighty percent DNA homology exists between these strains (Huang *et al.*, 1976).

1.2.1. Biological Properties.

Several properties are common to all cytomegaloviruses. These include: high species specificity, tight association with the host cell, low to intermediate virus yields and a slow cytopathic effect (CPE) (Rapp, 1984). In the human host, HCMV infects a variety of cell

types and is thought to enter both non-productive (non-permissive) and productive (permissive) cells. Non-productive infection is considered to favour the establishment of virus persistence and/or latency, a condition common to herpes viruses. Cytomegaloviruses also produce a distinctive CPE of cell enlargement (cytomegalia) with characteristic intranuclear and cytoplasmic inclusions which develop late in infection (Albrecht *et al.*, 1980).

1.3.1. **Replication.**

The reproductive cycle of cytomegaloviruses is slow, exceeding 24 hours. Adsorption and penetration of HCMV is a rapid process, and has been estimated to be approximately 3 mins for WI-38 fibroblasts (Smith and de Harven, 1974). Penetration is achieved either by adsorptive pinocytosis or by fusion of the virus to the cell membranes. Virus replication takes place in the nucleus, with the onset of DNA synthesis at approximately 12 hrs after infection (Goodheart *et al.*, 1960).

The relationships between the genetic organisation of the genome and the regulatory steps involved in the replication of the virus are still poorly understood. The expression of the HCMV genome is thought to be regulated in the infected cell (Stinski, 1984). It has been suggested that the regulatory factors may be virus as well as host specified (Stinski, 1984, Sissons *et al.*,1986). The physiological state of cells has been shown to affect their response to HCMV, and the production of infectious virions (de Marchi and Kaplan, 1977).

Subconfluent, actively growing cells have been shown to yield higher amounts of infectious virus than confluent-contact-inhibited cells. Higher yields of infectious virus has also been correlated with a greater percentage of cells producing viral antigens within the first 48 hrs after infection.

However, the nature of the functions required for the synthesis of viral antigens that are provided by cells in subconfluent-cultures but not in confluent cultures is unclear. What is known is that functions required for growth of HCMV are not those provided by cells in S phase (de Marchi and Kaplan, 1976). Gene expression in HCMV is temporally controlled, and is divided into immediate early, early and late stages, based upon the onset of virus specific protein synthesis and DNA replication.

1.4.1. **HCMV Persistence.**

Once an individual has been infected with HCMV, the virus is conserved for life in a latent or persistent form. The HCMV genome has evolved to remain in human cells from the time of primary infection, and although the majority of humans carry this virus, overt disease is rare.

Several authors have proposed that early gene expression, but not late gene expression occurs in non-productive cells, and that it is these cells that persistently harbour the viral genome (Stinski, 1978; Mocarski and Stinski, 1979 ; Wathen, *et al.*, 1981; Tanaka *et al.*, 1986). Reactivation of the HCMV genome in persistent infections allows for replication of the viral DNA genome and subsequent release of infectious virus to cause overt disease (Mocarski and Stinski, 1979). However, the mechanisms of reactivation are still poorly understood.

1.5.1. **Medical Importance.**

In normal individuals, a primary infection with HCMV is usually clinically inapparent, but can produce a mononucleosis-like syndrome accompanied by fever, hepatitis, fatigue and circulating atypical lymphocytes (Osborn, 1981; Ho, 1982). The severity of HCMV infection is realised in foetuses and immuno-compromised patients, usually allograft recipients or patients with immuno-suppressive diseases like HIV.

Cytomegalovirus can be isolated from most body fluids and secretions. It can be transmitted *in utero* despite the presence of high maternal antibody titres and can lead to congenital malformation resulting in mental retardation and deafness (Ho, 1982). It is responsible for about 7% of all infectious mononucleosis, causes 20% of graft rejections in renal transplant recipients and 25% of all mortality in this group. It also causes interstitial pneuomia which causes a 15% mortality rate in bone marrow transcipients.

Other syndromes associated with HCMV infection include cytomegalic inclusion disease, prematurity, intrauterine death and post perfusion syndrome (Ho, 1982). These diseases are usually not caused by primary infection, but are nearly always a result of reactivation of latent or persistent virus which is usually associated with immuno-suppression. Indeed, HCMV has been shown to have an immuno-suppressive effect on the cell-mediated immune system (Section 1.6.4).

1.2. Human Cytomegalovirus Structural Proteins.

The HCMV genome has an apparent coding capacity for 70-80 proteins. One hundred and thirteen structural polypeptides have been reported in the literature to date (Landini and Michelson, 1988) (Table 1). These proteins can be grouped in several ways depending on their function, locality in the virion and/or infected cell, time of appearance and immunogenic properties (Landini and Michelson, 1988 and Rasmussen, 1989).

1.2.1. Intracellular Virion Proteins.

The synthesis of HCMV particles within the infected cell has been characterised by biochemical fractionation procedures (Gibson, 1981; Imiere and Gibson, 1983,1985). Four different types of particles have been identified on the basis of their sedimentation properties in rate velocity sucrose gradients, intracellular compartmentalisation, protein composition and infectivity. By analogy with particles for HSV, HCMV particles have

been alphabetically designated as A, B, C and D. Within the nuclear fraction of infected cells, capsids A and B are found. At least three protein species of M.W. 145, 34, and 28 kDa have been identified in A capsid forms.

The 145-155 kDa protein is the major structural component of the icosahedral capsid, constituting 90% of its mass, and the 28 kDa protein has been shown to be important in maintaining structural integrity (Gibson, 1981). Capsid A forms are thought to be precursors of DNA containing B and C capsids and ultimately virions. B capsids contain proteins of M.W. 145, 45, 36, 34, and 28 kDa. The 36 kDa protein has been implicated in DNA packaging and/or nucleocapsid envelopment (Irmiere and Gibson, 1985). Enveloped B capsid forms referred to as non-infectious enveloped particles (NIEPs) also occur. C capsids contain two additional proteins of M.W. 205 and 66 kDa, whilst infectious virions have been shown to contain at least seven additional proteins not found in any of the capsid forms (Irmiere and Gibson, 1985). It has also been suggested that B-capsids are virion precursors, and that their maturation involves the elimination or modification of the 36 kDa "assembly protein".

1.2.2 Extracellular Virion Proteins.

Three distinct species of extracellular enveloped particles have been identified, these are:
(i) complete infectious particles which contain DNA, capsid, matrix protein and envelope.
(ii) non-infectious enveloped particles. These particles are structurally and compositionally similar to virions but contain no DNA.
(iii) dense bodies, which consist primarily of protein and contain no DNA or capsid. Dense bodies usually occur as large enveloped spherical aggregates (Irmiere and Gibson, 1983).

Table 1: Structural Proteins of HCMV

Protein (M.W.)	Glycoprotein	Phosphoprotein
>200	+	
212-200		
195-190	+	
185-180		
175-171	+	+
165-160	+	
155-153		
150-149		+
145-140	+	
135-132	+	
130-126	+	
122-120		+
116-115	+	
112-110	+	+
107-105	+	
100-90	+	+
98-93	+	+
92-91	+	
90-88	+	+
86-84	+	
83-79	+	
78-77		
76-75		+
74-73		+
71-69	+	+
68-66	+	+
65-64	+	+
63-62	+	

Table 1: **Structural Proteins of HCMV**

Protein (M.W.)	Glycoprotein	Phosphoprotein
61		
59-58	+	+
57		
55		
54		
53-52	+	+
51-49	+	+
48-47	+	
46	+	
45-44	+	+
43-42		+
40-39		+
38-37		+
36-35		+
34		
33-32		
31-29		
28-27		+
26-24		+
23-22	+	
21		
19-18	+	
17-16	+	
15		
14		
13-12	+	
11		

Proteins are given in molecular-weight size groups ($x10^3$). Proteins that are reported to be glycosylated or phosphorylated are listed.

Modified from: Landini and Michelson, 1988.

About 35 virus specific proteins have been detected in virions (Amadei *et al.*, 1983; Kim *et al.*, 1976; Gupta *et al.*, 1977; Stinski, 1977, 1978; Gibson, 1983). Some of these proteins have been shown to be glycosylated (Fiala *et al.*, 1976; Kim *et al.*, 1976; Stinski, 1976, Gibson, 1983). Furthermore, it has also been demonstrated that the glycoproteins which are soluble in detergents are highly immunogenic and associated with the virion envelope (Stinski, 1976; Furukawa *et al.*, 1984). Only the extracellular virion proteins that have been well characterised will be discussed. They, too, have been assigned to groups on the basis of their location within the virion and their function.

The viral matrix is the area located in the virion between the nucleo-capsid and the envelope and is present in virions, dense bodies and C capsid forms (Imiere and Gibson, 1983, 1985). The viral matrix protein, which is important in the assembly of HCMV, has been shown to contain at least 3 components, the lower matrix protein of 64-69 kDa, the upper matrix protein of 69-71 kDa (both these proteins are probably represented in the C capsid forms, and have also been designated as the 66 kDa protein) and the 150 kDa basic phosphoprotein (also referred to as the 145 -150 kDa icoshaedral capsid protein).

The lower matrix protein is phosphorylated and has variously been referred to as the 69 kDa matrix like protein (Irmiere and Gibson, 1983), pp65 (Nowak *et al.*, 1984), HCMV p64 (Clark *et al.*, 1984) and ICP27 (Geballe *et al.*, 1986). It is also the most abundant protein synthesised during the infectious cycle, and is a late gene product transcribed in the absence of viral DNA replication, which implies that it may be controlled by post-transcriptional events (Geballe *et al.*, 1986).

Although the lower matrix protein can be isolated in large amounts from the nucleus and cytoplasm of infected cells, its locality in extracellular virus forms is uncertain. It has been reported as an internal non-glycosylated phosphoprotein, inaccessible to antibodies in virions or on the infected cell surface (Britt and Vugler, 1987). Contrastingly, it has

also been claimed to be absent in virions, and only detectable in cytoplasmic dense bodies (Gibson and Irmiere, 1984; Kim, *et al.*, 1983; Landini, *et al.*, 1987).

The upper matrix protein, also referred to as the 74 kDa protein (Roby and Gibson, 1986) is not as abundant in the infected cell as the lower matrix protein (Gibson, 1983, Irmiere and Gibson, 1983). The 150 kDa basic phosphoprotein has been mapped to the Hind III J and N fragments and sequenced for strain AD169 (Jahn *et al.*, 1987). Two proteins of 67 and 68 kDa (probably the lower matrix protein) have been identified as phosphorylated protein kinases, and are present in all enveloped extracellular virion particles (Roby and Gibson, 1986). The 67 kDa protein has been localised to the Hind III F fragment and the 68 kDa protein to the Hind III L,b,c fragment (Landini and Michelson, 1989). The same authors have also indicated that pp65 (Jahn *et al.*, 1987; Rueger *et al.*, 1988) gp64 (Zaia *et al.*, 1984) gp66 (Kim *et al.*, 1983) and pp66-69 (Roby and Gibson, 1986) all correspond to the 68 kDa protein.

An abundant protein of 46 kDa present in extracellular virions has been characterised as a DNAse. The 28 kDa phosphoprotein is another abundant structural protein, localised to the surface of the viral capsid (Landini *et al.*, 1987). The 28 kDa phosphoprotein is highly immunogenic, and has been mapped to the Hind III R fragment.

1.2.3. **HCMV Envelope Proteins.**

The production of extracellular infectious virions starts in the nucleus of the host cell with the formation of icosahedral nucleocapsids. The nuclear capsids then acquire an envelope, which has been reported to be derived from both the internal nuclear membrane (Smith and De Harven, 1973) and from the endoplasmic reticulum (Severi *et al.*, 1979). The observation that antibodies directed against HCMV glycoproteins are reactive with corresponding antigens on internal and external membranes of infected cells supports this hypothesis (Stinski, 1976; Stinski *et al.*, 1979). However, Farrar and Oram, 1984, have

demonstrated that two distinct envelopes exist, an outer loose fitting envelope, which is sensitive to osmotic shock, and another osmotically stable envelope closely attached to the nucleo-capsid. Furthermore, intracellular virus particles isolated from infected cells were shown to possess only a single osmotically stable envelope, suggesting that the virions acquired their outer envelope as they leave the infected cell. It was also demonstrated that dense bodies did not contain the outer envelope, and also showed little or no surface structure.

SDS PAGE analysis of the polypeptides contained within the outer osmotically fragile envelope and the Triton X-100 extracted inner envelope indicated proteins with M.W. of 52, 95 and 130 kDa and 23, 30, 52, 67, 95, 130 and 250 kDa, respectively. Analysis of the Triton X-100insoluble fraction indicated that three proteins of M.W. 35, 64 and 150 kDa were associated with the nucleo-capsid (Farrar and Oram, 1984). The latter two proteins probably represent the lower matrix protein and the basic phoshoprotein respectively.

Virion envelope glycoproteins are considered important since they mediate viral entry, are involved in release of the virion from the host cell, and are the targets for neutralising antibody. They have also been shown to be important antigens in both cellular and humoral immune responses, and as such, potential candidate proteins for a sub-unit vaccine.

Five glycosylated components of M.W. 52, 67, 95, 130 and 250 kDa have been identified from purified virion envelopes treated with sodium metaperiodate and labelled with sodium borohydride (Farrar and Oram, 1984). These results were confirmed with further treatment of virus with galactose oxidase, and it was also shown that gp67 and gp130 contain relatively large amounts of galactose and or N-acetylgalactosamine residues. Glycoproteins of the following M.W. 210, 100, 62 and 57 kDa were shown to occur in both virions and dense bodies of CMV strain AD169 (Fiala *et al.*, 1976).

1.2.4. **Immunogenic Virion Proteins.**

The characterization of immunogenic proteins of purified virions has been undertaken primarily by immunoblotting and immunoprecipitation of HCMV sera from patients or sera raised against virions and virion envelope components (Farrar and Oram, 1984; Jahn *et al.*, 1987; Landini *et al.*, 1986; Re *et al.*, 1985). The number of polypeptides recognised with human sera and the intensity of the reaction have been found to correlate closely with serum IgG titres, as determined by enzymatic immunosorbent assays (Landini *et al.*, 1985). Virion proteins of M.W., >200, 150-155, 110, 99, 89, 82, 77, 74, 66-67, 61-62, 57, 55, 49, 38, 36, 33, 28 and 11 kDa react with HCMV IgG antibodies whilst virion proteins of M.W. 150-155, 64-66 and 34-38 kDa elicit an IgM antibody response. Some of these proteins have been shown to induce high titres of antibodies which remain circulating after the infection. By far, the most immunogenic protein described is the basic phosphoprotein of M.W. 150-155 kDa (Cremer *et al.*, 1985; Jahn *et al.*, 1987; Landini *et al.*, 1986). Antibodies to this protein also persist for years after convalescence, unlike the other immunogenic proteins (Landini *et al.*, 1986).

More recently, immunogenic virion proteins have also been identified by neutralising monoclonal antibodies reactive with proteins on the surface of virions. HCMV virion proteins with neutralising epitopes have been identified primarily with complement-dependent and independent murine monoclonal antibodies (Britt, 1984; Britt and Auger, 1985; Furlini *et al.*, 1987, Kari *et al.*, 1986; Pereira *et al.*, 1982, 1984; Rasmussen *et al.*, 1984, 1985a). Neutralising human monoclonal antibodies reactive with virion proteins have also been produced (Mashuo *et al.*,1987; Redmond *et al.*, 1986).

Three immunologically significant virion envelope proteins will be discussed in detail.

The gB complex of glycoproteins is considered to be the major set of immunogenic proteins for the virus envelope. It has been variously referred to as p130/55 (Rasmussen *et al.*, 1985), gA (Pereira *et al.*, 1982, 1984) and gCI (Gretch *et al.*, 1988a,1988c). The gene products are referred to as the gB complex because of its genetic homology with the gB gene of HSV I (Cranage *et al.*, 1986).

The gene coding for the gB complex was first mapped to the right terminal sequence of the Hind III F fragment between map coordinates 0.344 and 0.380 of HCMV (strain AD169) virion DNA (Mach *et al.*, 1986). A cDNA library was constructed from poly A+ RNA of HCMV infected λ gt 11 cells, and screened with monospecific rabbit antisera against the gB. The sequence for the gene was subsequently defined by Cranage *et al.*, (1986.)

This was achieved by the identification of a putative glycoprotein gene within a 20kb Hind III F fragment of HCMV. The predicted translation product of this gene with glycoprotein genes of other herpes virus showed homology with glycoprotein B(gB) of HSV, EBV and gpII of VZV, indicating that this gene is highly conserved among herpesviruses (Emini, 1987). It is also closely related to pseudorabies virus gII gene (Robbins *et al.*, 1987) and equine herpes virus (Whalley *et al.*, 1989).

Furthermore, sequence comparisons of HCMV-gB, HSV-gB and EBV-gB showed complete conservation of all 10 cysteine residues present between the putative signal and anchor sequences, suggesting that the extracellular portion of the proteins could possess a similar overall structure (Cranage *et al.*, 1986).

These findings were confirmed by sequence analysis of HCMV-gB for the Towne strain by Spaete *et al.*, 1988. The HCMV (Towne) gB gene was shown to have a 94% nucleotide similarity and a 95% amino-acid similarity to HCMV (AD169) gB gene, as described by Cranage *et al.*, (1986). Transcriptional analysis of the HCMV Towne gB-

coding strand indicated that the 3.9Kb gB message is transcribed as early as 4 hrs post-infection, and well in advance of gB protein synthesis (Spaete *et al.*, 1988), and as such, suggests that the gB gene may indeed be regulated at a post-transcriptional level. It has also been demonstrated that the gB glycoprotein is not expressed in the presence of phosphonoformic acid (PFA), confirming that it has the characteristics of a late gene product (Rasmussen, 1989).

The size of the gB protein was predicted from the 2721 bp open reading frame and is estimated to be 907aa. Hydrophopilicity:hydrophobocity index analysis of the amino-acid composition indicate a potential signal sequence of 24aa, and two potential transmembrane regions composed of 34aa and 21aa near the C terminus. The primary translation product is 102 kDa and cleavage of the signal sequence would result in a 99.4 kDa non-glycosylated protein. The gB (Towne) gene was also shown to have 20 potential N-linked glycosylation strain sites, as compared with the 16 or 17 reported for gB of AD169 (Cranage *et al.*, 1986). These differences could well account for the broad range of protein molecular weights reported for the gB.

Full-length and truncated versions of the gB gene were expressed in COS cells using expression vectors where transcription was driven by the SV40 early promoter or the HCMV major immediate early promoter (Spaete *et al.*, 19898). Amino-acid sequence analysis of the N terminus of the 55 kDa viral glycoprotein gp55 demonstrated that gp55 is derived from gB(gp130) by proteolytic cleavage and represents the C terminal region of gp130. The fate of the N terminal half of gB in CMV-infected cells is not known. The cleavage enzyme has been shown to be cellular in origin (Spaete *et al.*, 1988; Britt and Vulger, 1989; Pachl *et al.*, 1989). There may, however, be alternate cleavage patterns of the precursor protein, since the molecular weights of the deglycosylated forms, when added together, exceed that of the polypeptide precursor (Gretch *et al.*, 1988a). This is further supported by the fact that the open reading frame for the gB gene predicts as many as seven dibasic protein recognition sites (Cranage *et al.*, 1986). As such alternative

cleavage of the gp130 may well explain the diversity of related products within this complex.

Expression of gB in transfected cells was detected by immunofluorescence and ELISA using virus neutralising murine Mab 15D8 (Rasmussen *et al.*, 1984). The epitope recognised by Mab 15D8 was localised to within a 186 amino-acid fragment of the gp55 protein. Cells infected with recombinant-virus containing HCMV gB showed positive surface membrane fluorescence (Cranage *et al.*, 1986). The pattern of staining on the infected cell membrane was shown to be granular in appearance, and as such, suggested clustering or aggregation of HCMV gB in the cell membrane.

gB polypeptides synthesised in infected cells were characterised by immunoprecipitation of ^{35}[S]-methionine-labelled cell lysates with anti-HCMV gB monoclonal antibodies. Two polypeptides of 145 and 55 kDa were identified in infected cells whereas polypeptides of 130 and 55 kDa were detected in the mature virion for the gB Towne strain (Spaete *et al.*, 1988). Rasmussen, *et al.*, 1985a, reported infected cell proteins of 130 and 55 kDa and mature virion proteins of 130, 92 and 55 kDa to be reactive with Mab 15D8, one of the monoclonal antibodies used extensively in the aforementioned study. Pulse-chase experiment, to determine the relationship between the 145 and 55 kDa proteins produced in cells infected with recombinant-gB viruses, indicated that the majority of ^{35}[S]-labelled protein recognised by monoclonal antibodies reactive with gB was present as a 145 kDa protein, although smaller species of 66 and 55 kDa were also detectable.

Neutralizing murine monoclonal antibodies recognise HCMV proteins in virions and infected cells of similar electrophoretic mobilities to the gB proteins (Britt, 1984, Peirera *et al.*, 1984, and Nowak *et al.*, 1984).

The role of HCMV gB in virus infection is unknown. As with HSV-gB, HCMV-gB, has been shown to be the major component of the virion envelope (Law *et al.*, 1985), and a target for antibody-mediated neutralisation (Farrar and Oram, 1986). HSV-gB has, however, been implicated in viral entry and cell fusion, using temperature sensitive mutants. More recently, with the use of HSV-gB null mutants, gB has been shown to be required for viral infectivity in a stage after viral attachment, but before the expression of virus specific proteins (Cai *et al.*, 1988). It has also been demonstrated that gB of HSV contains different functional regions for fusion induction and inhibition. It has also been suggested that the 55 kDa protein of the gB of HCMV is located on the surface of the infected cell (Cranage *et al.*, 1986, Spaete *et al.*,1988). However the exact association of proteins that constitute the gB glycoprotein complex with the plasma membranes of infected cells has not been characterized.

Glycoprotein gH (Pachl *et al.*, 1989) also referred to as gC III (Gretch *et al.*, 1988c) is an envelope glycoprotein of M.W. 86 kDa (Rasmussen *et al.*, 1984). The gH gene has been localised to the Hind III L region of the unique long (U_L) segment of the HCMV (Strain AD169) genome (Gompels and Minson, 1986) and to the Hind III H region of the U_S region for the Towne strain (Pachl *et al.*, 1989). The gene was originally identified by characterisation of an open reading frame with a glycoprotein coding sequence. The predicted amino-acid sequence was shown to be homologous with the gH of HSV1 (Gompels and Minson, 1986), the BXLFZ gene product of EBV (Heineman *et al.*, 1988) and gp III of VZV (Keller *et al.*,1987). The gH gene has been cloned into a vaccinia virus expression vector and sequenced (Cranage *et al.*, 1988).

The gH glycoprotein is the target for complement independent neutralising antibody (Rasmussen, *et.al.*, 1984), and is also found in the cytoplasm of infected cells 24-48 hours post infection (p.i.) (Rasmussen *et al.*, 1984) and in infected cell plasma membrane extracts 72 hrs p.i.(personal communication P. Roig Farran). Unlike glycoprotein gB, gH has been shown to be a single gene product of approximately 80

kDa. High mannose-linked oligosaccharides make up the other 6 kDa. The gH glycoprotein has been implicated in other intracellular dissemination of the virus as well as in the early stages of virus infectivity. Anti-idiotype antibodies which bear the internal image of a neutralising epitope on the gH glycoprotein inhibits the spread of HCMV, and as such suggests that the gH glycoprotein interacts with cellular receptors (Keay *et al.*, 1988). Monoclonal antibodies to an 85 kDa glycoprotein of EBV (the gH equivalent) inhibits cell fusion, important in the spread of the virus from cell to cell, but does not inhibit cell attachment (Miller and Hutt-Fletcher 1988).

Virion envelope glycoprotein complex gCII is coded from the HXLF multigene family (Gretch *et al.*, 1988b). The HXLF gene family has five genes that share one or two regions of homology and are arranged in tandem within the unique short (U_S) component of the HCMV genome (Weston and Barrel, 1986). The homologous ORF's are thought to be the result of gene duplications and expansions of the U_S region. It contains two unreduced proteins of M.W. 93 kDa and 450 kDa. After reduction, two protein components of M.W. 50-52 kDa and >200 kDa, and minor components of M.W. 90 kDa, 116 kDa and 130 kDa are detected (Kari *et al.*, 1986).

Peptide maps of the 50-52 kDa, 93 kDa and 200 kDa species of the gCII complex of proteins indicate that they are structurally similar. The glycoproteins of this complex have also been shown to be heavily sialated and the 47-52 kDa protein to contain high amounts of 0-linked oligosaccharides in comparison to the other proteins. A precursor protein of 25-32 kDa has also been identified for glycoprotein 47-52 kDa. The amino-acid composition of the 47-52 kDa glycoprotein has been shown to be most similar to the predicted amino acid composition of the HXLF 2 and HXLF 1 ORF.

1.3. **HCMV Infected Cell Proteins.**

Infection of cells with HCMV, even at high multiplicities of infection, causes an increase in host cell DNA, RNA, and protein synthesis above the level of that in most uninfected cells (Furukawa *et al.*, 1973; Stinski, 1977). In cell cultures infected with HCMV, there is an initial decrease in the rate of protein synthesis which is associated with virus adsorption (Garnett, 1979), and then this is followed by an increase at two different periods after infection (Stinski, 1977).

Generally, protein synthesis during the early phase of HCMV infection represents 70% of host cell protein synthesis, with virus specific protein synthesis increasing at a linear rate after the initiation of viral DNA replication, i.e., at 10 hrs p.i. (Stinski, 1978). At 48 hrs, p.i. virus specific protein synthesis represents approximately 60% of total protein synthesis and remains at this level for as long as 5 days. As such, it is difficult to fully differentiate between virus specific and virus induced host cell proteins. Indeed, biochemical and immunological analysis of proteins from purified virions indicates the presence of host cell proteins within the virion (Mar *et al.*, 1981; Michelson *et al.*, 1989). Infectious virus production is detectable 48-72 hours p.i., and it is presently assumed that the slow rate of HCMV DNA replication influences the relatively slow production of infectious virus. This may, in turn, be related to the propensity of the virus to establish persistent infections. HCMV proteins have been classified into "immediate early", "early", and "late proteins" based on the cascade regulation of protein synthesis observed in infected cells with HSV 1. Early proteins have been defined as all proteins made before the onset of DNA replication and late proteins after DNA replication.

However, it has been demonstrated that several late structural proteins are transcribed and translated before viral DNA synthesis albeit at a very low level (Mocarski *et al.*, 1985). Numerous transcripts coding for late gene products have been shown to appear early in infection, but little or no RNA can be found in the cytoplasm at this time (Wathen and Stinski, 1978). Conversely, several immediate early and early proteins are known to persist during the late phase of virus infection (Michelson *et al.*, 1979).

Immediate early (IE) proteins are implicated in the establishment of virus infection and have been shown to be phosphorylated proteins (Gibson, 1981; Cameron and Preston, 1981), DNA binding proteins (Gergerly *et al.*, 1980) and proteins which cause alterations in host cell chromatin structure and template activity (Kamata *et al.*, 1978, 1979). The majority of the IE genes are transcribed from a restricted region of the long unique (U_L) sequence contained within the Hind III E fragment (Sissons *et al.*, 1986). The number of IE proteins reported for the infected cell varies as do the molecular weights (see Table II).

In vitro translation of the immediate early genes gives rise to at least 7 proteins (Wathen *et al.*, 1981). Some of the IE proteins are only transiently expressed while at least one of M.W. persists throughout the replicaton cycle (Michelson *et al.*, 1979). Some of the IE proteins have also been shown to activate genes in trans. In short-term transfection assays, the Hind III E fragment of HCMV DNA produces trans activation of transcriptional units from both viral and cellular promoters (Sissons *et al.*, 1986). In HSV infection, the major IE proteins act as transacting factors and initiate the transcription of the early virus proteins.

The alteration in transcription of host cell genes by IE proteins of HCMV is implicated in the cause of some of the pathogenic effects of HCMV (Sissons *et al.*, 1986). This IE gene enhancer/promoter unit of HCMV is unique in its complexity and transcriptional strength in several cell types. Besides a unique region, it also contains a number of different repeat units of 17-, 18-, 19- and 21- base/pairs which act as targets for cellular trans-acting factors. The 19 bp has been shown to be essential for constitutive activity of the enhancer, and the inner palindromic core of the 19 bp unit also corresponds to C-AMP response elements (CRE's) contained within various hormone genes and the C-fos gene (Stamminger *et al.*,1989).

Table II. **Immediate Early Proteins Induced by Different Strains of HCMV**

AD-169	Davis	Towne
Proteins detected in infected cells		
110		110
79	78	78
78	70	76
77		72
73		68
71		
57		56
53		52
38		38
31		30
in vitro translation products of IE genes		
Region 1	Region 2	Region 3
75	75	68
39	56	
	42	
	21	
	16.5	
	6.59(?)	

The molecular weights (x 10^3) of proteins reported to be induced by strains AD-169, Davis and Towne are given.

From Landini and Michelson, 1988.

Other cellular factors have also been implicated in the initiation of transcription from this region. The interaction of necrosis factor one (NF1) with the 18 pb unit has been shown to mediate the activation of IE gene expression in phorbol ester-mitogen stimulated T-lymphocytes, in the absence of any viral transactivators (Cherington *et al.*, 1989).

It has also been suggested that cells which are non-permissive for HCMV infection contain differentiation specific factors that repress IE gene expression (Shelbourn *et al.*, 1989). These authors have demonstrated that binding factors specific for the 17, 18, 19 and 21 bp repeat units and the NF1 binding site of the major IE regulatory region are present in undifferentiated human teratocarcinoma cells (T2 cells), and that retinoid acid induced differentiation of T2 cells to a HCMV permissive phenotype can be correlated to major changes in the presence of specific binding proteins to some but not all of these sequences.

To date, the 19 bp repeat unit has been shown to be the only element shared among CMV enhancers, and as such, suggests that it plays a key role in the life cycle of the virus. Finally, it has been shown that expression of IE proteins does not take place in some latently infected cells (Gonczol *et al.*, 1985; Mocarski *et al.*, 1979).

Early (E) proteins are transcribed approximately 2 hrs p.i. and well in advance of DNA synthesis (Blanton and Tevethia, 1981). Early proteins synthesis has primarily been studied in permissive cells in which viral DNA synthesis has not occurred (Blanton and Tevethia, 1981 Stinski, 1977, 1978) or is blocked with inhibitors of viral DNA polymerase such as phosphonoacetic acid (PAA) or phosphonoformate (PFA) (Stinski, 1978). Infected cells non-permissive for HCMV DNA synthesis have also been used to study early proteins (Stinski, 1978).

To date, approximately, 25 early proteins have been described (Table III), the majority of which are non-structural proteins and include DNA binding proteins (Anders *et al.*, 1986;

Table III. **Early HCMV Proteins**

M.W.Size	Characteristic
145-140	pol,GP, DB
133-130	host GP
129	DB
120-118	GP
114	GP
108	
100-97	
91	
88-87	
72-70	GP
68	PK,PP
59-58	pol
56	
53-52	DB
51-50	DB,PP
49	
46-45	DB
43	
41	
40-39	
38	
37-35	DB
27-27	DB
21	
19-18	DB
12	GP

Proteins are listed by molecular-weight size groups. Molecular weights (x 10^3) are given. Properties of the proteins (pol = DNA polymerase; GP = glycoprotein; DB = DNA-binding protein; PK = protein kinase ; PP = phosphoprotein).

From Landini and Michelson, 1988.

Gergerly *et al.*, 1980, 1985; and Gibson, 1983) phosphoproteins (Mocarski *et al.*, 1985) glycoproteins (Stinski, 1976) and enzymes (Michelson *et al.*, 1984; Huang, 1986).

However, it is not known whether all these proteins are indeed coded for by the HCMV genome or are novel virus-induced host proteins. The synthesis of a virus-induced host cell glycoprotein of 130 kDa has been reported to be necessary for viral DNA synthesis (Radsak *et al.*, 1985). Late proteins are made after the synthesis of viral DNA, and are considered to be virion structural proteins and have been described in Section 1.2.

1.4. HCMV-Induced Plasma Membrane Proteins.

Based on studies with antisera produced against membranes of HCMV infected cells, it has been demonstrated that the plasma membranes of infected cells contain virus specific antigens as early as 24-48 hrs post-infection (Stinski *et al.*, 1979). During the course of infection, these viral antigens accumulate in the plasma membranes, endoplasmic reticulum and nuclear membranes (Stinski *et al.*, 1979).

Biochemical and compositional analysis of changes in the plasma membranes of fibroblasts infected with HCMV has shown that approximately 12 novel plasma membrane proteins are detected during the course of infection (Sullivan-Tailyour and Garnett, 1986). The molecular weights of these proteins range from 24-26 kDa to 260-270 kDa (see Table IV). Glycoproteins of M.W. 53-55, 60-63, 70-72, 98-103 kDa and proteins of 130-133K and 260-270K kDa were shown to be most abundant. Glycoproteins of M.W. 60-63, 70-72 and a protein of 130-133 kDa were detected in the plasma membranes at 8 hrs p.i., and the remainder of the proteins 48-72 hrs p.i. These proteins are likely to be part of either the gB/gCI (Cranage *et al.*, 1986 ; Spaete *et al.*, 1989; Gretch *et al.*, 1988) or gC II (Gretch *et al.*, 1988) glycoprotein complexes, since several of the reported proteins are of similar molecular weights.

Table IV. Characteristics of the Novel Plasma Membrane Proteins and Glycoproteins of HCMV-Infected HEF

Mol. Wt. ($X 10^{-3}$)	Frequency of Detection and Time of Appearance		Protein Moiety Exposed 8h		Proteins Labelled with (^{3}H)arginine	Glycoproteins (mannose-labelled)	Glucosyl residue exposed	Serology* 1	2	3
24-28	+30%	72h	-	-	+	-	-	-	+	-
32-34	-		-	-	-	+	-	-	-	+
42-43	+20%	72h	-	-	+	-	-	-	-	-
53-55	+50%	48h	-	+	+	-	-	+	-	-
60-62	+60%	8h	-	+	+	+	-	+	-	-
70-72	+80-90%	8h	+	+	+	+	-	+	+	+
78-80	+20%	72h	-	-	-	-	-	+	-	-
92	+30%	72h	-	-	+	+	-	-	-	-
98-103	+80-90%	24-48h	-	-	+	+	-	+	-	-
130-133	+80%	8h	+	+	-	-	-	-	-	-
145-150	+30%	48-72h	-	+	+	+	+	+	+	+
160-162	-		-	-	-	+	-	-	-	-
188	-		-	-	-	-	-	-	-	-
205-215	+20%	72h	-	-	-	+	-	-	-	-
210	-		-	-	-	+	-	-	-	-
230-240	-		-	-	-	-	-	+	-	-
260-270	+80%	72h	-	-	+	-	-	-	-	-

* 1. Reacts with antibodies in human serum; 2. reacts with antibodies in antiserum to virions and dense bodies; 3. reacts with antibodies in antiserum to envelope glycoproteins.

From: Sullivan-Tailyour and Garnett, 1986.

1.5. Immunology of HCMV.

Antibodies appear to be an important protective factor in primary infections, and it is likely that their major role is to prevent a generalised spread of virus which can result in symptomatic infection (Apperley and Goldman, 1988: Rasmussen *et al.*, 1989). The antibody response in normal individuals following primary HCMV infections consists of an initial transient IgM response, which is then followed by persisting levels of IgG (Bettes and Schmidt, 1981; Sweet *et al.*, 1985). However, reactivation infections do not necessarily elicit an IgM response (Rasmussen *et al.*, 1982; Griffiths *et al.*, 1982; and Pass *et al.*, 1983).

HCMV proteins that elicit the formation of antibodies have been studied primarily by assessing the reactivity of human immune sera with viral proteins from either infected cells or purified extracellular virions (Blanton and Tevethia, 1981; Farrar and Oram, 1984; Landini *et al.*, 1985, 1987; Middeldorp *et al.*, 1985; Pereira *et al.*, 1983; and Sullivan-Tailyour, and Garnett, 1986). Furthermore the reactivity of purified viral proteins known to elicit neutralising antibodies *in vitro* with human immune sera has been assessed (Cremer *et al.*, 1985; Liu *et al.*, 1988).

Antigenic HCMV virion and infected cell proteins have been variously grouped on the basis of the progression of immune reactivity *in vivo*, time of appearance and locality within the infected cell, biochemical characteristics and immunological reactivity with known virion structural proteins.

In normal seropositive individuals, antibodies to at least 15 HCMV associated proteins are detected (Gold *et al.*, 1988b). Individual sera have been shown to exhibit different patterns of reactivity and the basis for this variability has been attributed to strain differences and antibody affinity, and differences in the reactivity to laboratory strains and clinical isolates. Patterns of "early, "late" and "variable" responses to specific groups of

HCMV-associated proteins have also been detected (Gold *et al.*, 1988a, 1988b). The "early" responses most frequently recognise proteins of M.W. 30, 35, 40, 50, 66 and 80 kDa, whilst "variable" responses have been designated to proteins of M.W. 42, 58, 63 and 115 kDa. Antibodies have also shown different specificities for polypeptides at different times after sera conversion (Cremer *et al.*, 1985). As such, the detection of "marker" antibodies may be useful in evaluating the time course and nature of an infection.

Immediate early proteins of M.W. 78-80, 72-76, 69-70, and 30 kDa from infected cells have been shown to react with sera from acutely infected individuals (Blanton and Tevethia, 1981). Early proteins of M.W. 118, 114, 108, 97, 91, 87, 68, 52, 51, 49, 46, 43, 41, 40, 38, 27 and 27 kDa have been shown to be recognised by HCMV immune sera (Blanton and Tevethia, 1981). Some of these proteins are reported to be located located in the membranes of infected cells (Boldogh *et al.*, 1977, Stinski *et al.*, 1979). Late proteins of M.W. 183, 150-155, 120-130, 110, 92-96, 82-86, 80, 71-74, 69, 64-66, 58-61, 49-55, 39-44, 34-38, 32-33 and 25-30 kDa have been identified in infected cell extracts using human sera. Several studies have been carried out which demonstrate that HCMV immune sera (Middeldorp *et al.*, 1985; Sullivan-Tailyour and Garnett, 1986) and neutralising monoclonal antibodies (Pereira *et al.*, 1982) recognise antigenic determinants present on the surface of HCMV-infected cells. It has also been shown that sera raised against HCMV-infected cell membranes reacts with extracellular virions and dense bodies (Stinski *et al.*, 1977).

Proteins of M.W. 145-150, 120-130, 110, 80, 64, and 28-30 kDa have been shown to be predominantly located in the infected cell nucleus (Yamauchi *et al.*, 1985) while proteins of M.W. 94, 88, 67 and 32-34 kDa are located in the cytoplasm (Zaia *et al.*, 1986). Furthermore, Sullivan-Tailyour and Garnett, (1986) indicated that it is highly likely that plasma membrane proteins of M.W. 34, 53-55, 60-63, 70-72, 98-103, 130-133 and 145-150 kDa could be HCMV-specified virion constituents. These proteins are

exposed on the cell surface and react with human HCMV-immune sera. Glycoproteins of M.W. 34, 70-72, 98-103, and 145-150 kDa were also detected by immunoblotting techniques with sera raised against virions (Stinski, 1977) and extracted envelope glycoproteins (Farrar and Oram, 1984). Although these proteins are constituents of the plasma membranes of infected cells, it is uncertain whether they eventually become incorporated into the virion envelope. Surface- labelled HCMV-infected cell extracts have also been shown to immunoprecipitate exposed plasma membrane proteins of M.W. 53-63, 94, and 94-120 kDa with HCMV antibody-positive sera from healthy individuals (van der Voort *et al.*, 1989). The same proteins were shown to react with sera from renal transplant recipients with active HCMV infections. Two other plasma membrane proteins that were shown to react with human sera are of M.W. 230-240 and 78-80 kDa (Sullivan-Tailyour and Garnett, 1986).

Of the 4 IgG subclasses, IgG_3 has been shown to be the most prevalent and is then followed by IgG_1 (Landini *et al.*, 1987). This is of particular interest in that IgG_3 constitutes only 5.3% of the total serum IgG content and is suggestive of a preferential induction of IgG_3 production during active CMV infection (Morell *et al.*, 1972). The preference for IgG_3 is primarily directed against proteins of M.W. 66, 61, 55, 49 and 38 kDa, and it has been suggested that the presence of antibodies against these proteins in patient sera could serve as markers for active HCMV infection (Landini *et al.*, 1987).

During convalescence, the reactivity of all four IgG subclasses is evident only against the basic phosphoprotein pp150 and phosphoprotein p28, while the intermediate M.W. polypeptides show variable IgG subclass reactivity. The HCMV structural polypeptide pp150 has been shown to be the most immunogenic, Landini *et al.*, 1985, 1986; Pereira *et al.*, 1982, 1983; Jahn *et al.*, 1987. A high antibody response (IgM and IgG) to p66 together with a low antibody response to pp150 is considered to be a characteristic feature of primary HCMV infection (Landini and Michelson, 1988). However, it has also been shown that antibody responses during congenital and perinatal HCMV infection show a

high reactivity for phosphoprotein 150-155 kDa, and it is only several months after birth that other virion proteins are detected in sera. Antibodies to glycoprotein gB have been shown to be present in serum samples from both primary and reactivated infections (Cremer *et al.*, 1985).

HCMV specific antibodies with neutralising activity, as well as complement fixing ability are detected in sera after acute infection (Booth and Mohammed, 1988; Ho, 1982). The extent to which these antibodies serve to limit HCMV replication is unknown. However, it has been suggested that HCMV specific immunoglobulins are likely to exert their greatest antiviral role while functioning interdependently with Fc receptor-bearing lymphocytes to mediate antibody dependent cell-mediated cytotoxicity against infected cells (Quinnan *et al.*,1982). Further, cytolytic antibodies are detected in both acute and convalescent sera of patients (Middeldorp *et al.*, 1986). Antibodies (IgM and IgG) from HCMV hyperimmune sera lyse CMV-infected cells in the presence of guinea pig complement (Betts and Schmidt, 1981; Middeldorp *et al.*, 1984). The target antigens for cytolytic antibodies are poorly defined. Antigens present on the surface of infected cells early and late in infection are involved and it is not known whether antigens present on the viral envelope play a role (Middeldorp *et al.*, 1986). However, antibody-mediated cytolytic responses have been shown to be negligible in patients with fatal HCMV infection.

The importance of cell-mediated immunity to HCMV is exemplified by the occurrence of severe and prolonged infections in individuals with congenital, saprogenic or acquired deficiencies of cellular immunity. In contrast, infection of most individuals with primary B-cell disorders is usually not severe.

In patients undergoing immunosuppressive therapy or with acquired immunodeficiency syndrome, who have profound defects in cell-mediated immunity, but normal levels of circulating antibody, symptomatic HCMV infection, such as, interstitial pneumonitis,

retinitis, colitis, hepatitis, and encephalitis are common. From these observations, it is presumed that the delayed hypersensitivity response and T-lymphocytes play a critical role for maintenance of the latent or limited replications that is characteristic of latent infection. However the role of specific antibodies, which may not be produced in these individuals, has not been assessed.

Never the less, the normal functioning of various populations of cytotoxic lymphocytes is considered important for complete recovery from HCMV infections (Meyers, 1984; Quinnan *et al.*, 1982; Rook *et al.*, 1984). However, it is also known that HCMV can infect and adversely alter the function of these and other critical immune cells critical immune cells (Buchmeier *et al.*, 1989).

Antigen specific T-helper cells have been shown to recognise processed HCMV antigen(s) in association with class II major histocompatibility complex (MHC) genes expressed primarily on the surfaces of macrophages, but also on other cell types, such as, activated B-cells, dendritic cells, human dermal fibroblasts and epidermal langerhans cells. It has been demonstrated that T-helper cells (CD4+) preferentially recognise antigens that are present on intact virus particles, whereas T-suppressor cells (CD8+) recognise antigens of HCMV infected cells, and demonstrate class I MHC restricted lysis (Borysiewicz *et al.*, 1983). Interestingly, EBV-transformed lymphocyte cell lines are able to present MHC restricted HCMV antigen to HCMV specific T-helper (CD4+) clones (Liu *et al.*, 1987).

It has also been shown that the products of all three MHC class II families, DR, DQ and DP function to restrict HCMV recognition (Linner *et al.*, 1986; Gehrz *et al.*, 1987).

The transfer of helper (CD4+) lymphocytes has been shown to restrict MCMV replication in the adrenal gland during acute infection of mice (Shanley, 1987). The primary

restricting determinants of these 3 families have been shown to function at a subtypic level, which are closely associated with single Dw specificities (Linner *et al.*, 1986).

Some HCMV proteins have been shown to induce T-lymphocyte proliferative responses (Charpentier *et al.*, 1986). The gB glycoprotein complex, is able to induce T-helper cell, blastogenesis, both *in vivo* and *in vitro* (Gonczol *et al.*, 1986, 1987), and an early protein of M.W. 72 kDa also stimulates helper T-lymphocytes and cytotoxic class I restricted CD8+ cytotoxic lymphocytes (Rodgers *et al.*, 1987).

The immunosuppressive effects of HCMV on T-lymphocytes, are as yet not fully understood. However, by *in situ* hybridisation techniques, it has been demonstrated that the HCMV genome is present in a higher percentage of T_4+ lymphocytes (2.4%) than T_8+ lymphocyte (0.8%) from healthy previously infected individuals (Schrier *et al.*, 1985).

Natural killer cells are considered to be important in limiting the severity of HCMV infection prior to the development of T-cell mediated immunity.

For example, in bone marrow transplant patients at high risk for HCMV infection the levels of natural killer cell cytotoxity for HCMV infected cells is considered to affect both the acquisition and outcome of HCMV infection after transplantation (Bowden *et al.*, 1987). It has been shown that patients whose peripheral blood mononuclear cells have the ability to lyse HCMV infected cell targets during the first 20-60 days after transplantation showed a delayed time period of acquisition of the virus.

Since the target structure of NK cells is thought to be of cellular and not viral origin, natural killer cells interact directly with the infected cell to bring about cell lysis. Their ability to kill a broad range of virus-infected cells independent of any known "immunological" specificity or memory is of importance to the initial stages of HCMV

infection, since they are able to curtail the amount of infectious virus produced early in infection.

The role played (if any) by infected cell glycoproteins or polypeptides located at the surface of the cell with respect to triggering a NK cell response is uncertain. However, since it has been shown that NK cells express a low affinity F_C receptor for aggregated IgG or CD16 antigen which is recognised by several monoclonal antibodies (Perussia *et al.*, 1983a and b).

1.7. Eukaryotic Cell Plasma Membranes: Isolation and Purification.

The cell surface membrane or plasma membrane is the physical boundary which separates the internal environment of the cell from the external environment. As such, the surface membrane is involved in a wide variety of cellular functions. All stimuli which the cell utilises as information must interact with this boundary, and likewise, all modifications that the cell exerts on its environment must cross or occur at the boundary of the cell. Procedures for obtaining surface membrane-enriched fractions for a wide variety of tissues and cell types have been described (Neville, 1976). Most methods utilise a combination of isopycnic density gradient centrifugation and rate sedimentation centrifugation. The average enrichment of a putative surface membrane biochemical marker referenced to the original homogenate for fibroblasts is 4-10X (Warren *et al.*, 1976; Brunett and Till, 1971). However, enrichment of up to 20-25X greater than the homogenate have been reported for plasma membranes isolated from other tissue types (Neville, 1976).

Although surface membrane-enriched fractions are easily obtainable, surface membrane fractions of documented purity are not common. It should also be emphasised that each cell type, tissue, species, and passage number, has its own characteristics of cell fragility

and ease of membrane vesiculation, or sheet formation, which dramatically affects plasma membrane sedimentation properties and renders the conditions of either homogenisation or methods of shearing membrane sheets from an adherent monolayer of cells a major variable.

Membrane purity can be assessed by morphological criteria when a distinctive surface membrane morphology exists, like large sheet-like membranes obtained by FMA extraction (Barland and Schroeder, 1970).

Quantitative documentation of purity requires specific activities in both the initial homogenate and final membrane fraction of markers associated with intracellular organelles that are most likely to be a source of contamination (Brunett and Till, 1971). Smooth vesicles derived from the endoplasmic reticulum (ER), provide the most difficulty, and represents the most frequent contamination (Hubscher and West, 1965). However, most preparations are reported to contain less than 20% of their membraneous material derived from ER, mitochondrial fragments and lysosomes.

The two most crucial steps in isolating plasma membranes are the techniques used to dissociate the plasma membrane from the cell, and the subsequent extraction of plasma membranes from the homogenate.

Ideal homogenisation conditions require a monodispersed homogenate. Essentially, the homogenisation procedure should leave the particulate organelles such as the nucleus, mitochondria, lysosomes and peroxisomes intact, whilst the golgi complex, plasma membrane and reticular organelles should form homogenous-sized vesicles with the same orientation as exists *in vivo*.

However, tissue culture cells present a `new' unique set of problems in cell fractionation with regard to the release of all organelles in suspension as individual elements and much

of the methodology developed for extracted tissues is inappropriate (Howell *et al.*, 1989). The major difference between extracted tissue and cultured cells is the differential organisation of the cytoskeleton in cultured cells which results in the cytoplasm maintaining some degree of organisation after homogenisation.

As such, the major obstacle to be overcome in tissue culture cells is the complete depolymerisation of skeletal elements. Using tissue homogenisation procedures with cultured cells, it has been found that organelles tend to remain associated with the cytoskeletal elements surrounding the nucleus, and become entrapped in clumps of cytoplasm, which readily sediment. Up to 50% of the components of the homogenate may, therefore, be pelleted along with the nucleus during the initial centrifugation of plasma membrane.

In order to prevent this from taking place, the parameters that influence the efficiency of homogenisation of cultured cells have to be rigorously standardised. These include: growth and experimental conditions of the cells, homogenisation media, disruptive agents, and homogenisation devices. Cultured cells that have been growing in a confluent state for a number of days have a more complex network of cytoplasmic filaments, and are more difficult to process (Franke *et al.*, 1981).

However, optimum homogenisation can be achieved with cells that are 12-36 hours post passage, the precise time depending upon the density of cells required. The ideal concentration of cells for homogenisation has been estimated to be between 2.6-5.2 x 10^7. Pre-treatment conditions of the cells before homogenisation causes changes in the cells, such that different homogenisation conditions have to be used. Cells grown in monolayers may be brought into suspension either by trypsin treatment, scraping directly from the tissue culture flask or cold treatment with 1mM EDTA. In the latter case, these cells have to undergo significantly harsher homogenisation conditions than those cells treated by scraping. Cells that have been treated with trypsin, have to be left

for a sufficient time period before homogenisation to allow for the restoration of cell surface molecules that may have been cleaved by proteolysis. (Howell *et al.*, 1989).

To maintain the structural integrity of plasma membranes, isotonic conditions at neutral pH are required. This has been shown to be essential for the retention of the fluid phase content within the lumen of the vesicles derived from organelles of the secretory and endocytic pathways.

Homogenisation in isotonic buffers does not interfere with standard biochemical enzymatic of immunological assays (Esmann, 1988, Grivell *et al.*, 1986, Hubscher and West, 1965). However, some cells are difficult to fractionate in isotonic buffers, and it has been demonstrated that in these circumstances the forces required to break the cells also disrupts the nuclear membrane resulting in the release of DNA. This causes the cytoplasm to clump and prevents further separation (Howell *et al.*, 1989).

Imidazole (3mM) or Tris(hydroxymethyl)aminomethane (10mM)/acetic acid buffers have proven effective homogenisation buffers for the above conditions, but it should be noted that isotonic conditions should also be restored as soon as possible (Howell *et al.*, 1989). Proteolytic inhibitors are usually included in the homogenisation buffer. The choice of which is largely empirical. The following have been used, 1mg/ml pepstatin, 1mg/ml leupeptin, 1mg/ml apronitin, 1mg/ml antipain, 17mg/ml benzaminidine (Howell *et al.*, 1989).

Depolymerisation of the filaments of the cytoskeletal network microtubules, microfilaments and intermediate filaments have been shown to improve the quality of the homogenate from cultured cells. Microtubules can be depolymerised with nocodazole or colchicine and actin containing microfilaments with cytochalasin (Howell *et al.*, 1989). The presence of these drugs in the homogenisation buffer does not necessarily result in a positive improvement in the quality of homogenate. However, these drugs have not been

extensively tested and it has been shown that microtubules are probably depolymerised, by either prolonged periods at 4°C or by dilution. It is the intermediate elements that are thought to present the greatest problem. They form a distinct whorl around the nucleus and are also dispersed throughout the cytoplasm individually and in bundles (Franke *et al.*, 1981). To date, no methods to depolymerize the intermediate elements under non-denaturing conditions are available.

High salt conditions (1M KCl) have been used to disaggregate vesicles and prevent redistribution of luminal proteins. However, these conditions have been shown to promote the condensation on intermediate filaments leading to increased aggregation. Trypsin treatment of the post-nuclear supernatent fraction, (0.5-.10 mg/ml for 3-5 min at 37°C) followed by immediate cooling and addition of 20 mg/ml soybean trypsin inhibitor, has been found to increase the efficiency of plasma membrane separation. Trypsin exerts its effect by disrupting the interaction between the cytoplasmic filaments Howell *et al.*, 1989)

Plasma membrane isolation and purification procedures can be broadly divided into two categories, those that require cell homogenisation prior to plasma membrane purification and those that allow for direct extraction of plasma membrane.

A widely used method for the isolation of plasma membranes from mammalian cells involves sonication or homogenisation of cells followed by the separation of plasma membranes by centrifugation in discontinuous sucrose gradients (Allan and Crumpton, 1970; Lunstra *et al.*, 1974). However, since it has been shown that cells and cell organelles are highly susceptible to damage by sucrose gradients, the likelihood of the plasma membrane fraction being contaminated with membranes from various cellular organelles is high. The method of nitrogen cavitation which allows for the specific dissociation of plasma membranes from cells without affecting other cell structures followed by separation of these membranes by discontinuous sucrose gradient

centrifugation is reported to give a higher yield and purity compared to conventional sucrose gradient methods (Peterson *et al.*, 1980).

Plasma membranes has also been isolated by two-step differential centrifugation in the presence of Ca^{2+} (Liu *et al.*, 1987). This method is said to confer a major advantage over that of the previously mentioned methods in that membranes from relatively small numbers of cells, can be purified. Calcium, in the form of $CaCl_2$ (10mM) is used to precipitate out any microsomal contamination after cell homogenisation. The presence of millimolar Ca^{+2} selectively aggregates microsomal membranes. Although the mechanism(s) allowing for selective aggregation are poorly understood, it has successively been used to eliminate microsomal contamination.

A procedure that allows for the direct isolation of plasma membranes without homogenisation is the isolation of cytospheres from liver parenchyma cells by centrifugation at 100,000g. This causes the cells to exude vesicle-like bodies called cytospheres (Grivell *et al.*, 1986). Cytospheres possess a tri-laminar membrane structure typical of plasma membranes and a finely granular hyaloplasm generally devoid of organelles, filaments and microtubules. Ouabain and wheat germ agglutinin binding studies have indicated that the original orientation of the plasma membrane is also maintained throughout the formation of cytospheres. Furthermore, lipid analysis of the cytosphere membranes has indicated that they are indeed derived from the plasma membrane of the cell (Grivell *et al.*, 1986).

Aqueous two-phase systems resulting from an incompatibility between aqueous solutions of two polymers, polyethylene glycol and dextran, have been used for the resolution of a variety of macromolecules and cellular organelles (Klockman and Deppert, 1983). The two-phase method, one of the plasma membrane isolation procedures used in this study, permits sedimentation of the plasma membranes at the interphase where surface tension is minimal, allowing for a relatively undisturbed membrane structure.

Plasma membranes from mammalian cells have been isolated by 2-phase polymer systems from the following sources: L-cells (Brunett and Till, 1971), chick choriallantoic cells and fibroblasts (Israel *et al.*, 1973), and goat epididymal spermatozoa (Rana and Majumder, 1987).

Factors influencing the yield and purity of primary cultures of fibroblasts plasma membranes isolated by aqueous two-phase polymer systems are: (i) the conditions of hypotonic shock used to dissociate the plasma membrane from the cells, (ii) the molecular sizes of the dextran and polyethylene glycol that constitute the two-phase polymer system, and (iii) the centrifugal forces used for the sedimentation of membrane at the interphase.

The other method of plasma membrane isolation used in this study was the fluorescein mercuric acetate (FMA) procedure of Barland and Schoeder, 1971. This procedure allows for the direct isolation of surface membranes from adherant monolayers of fibroblasts. No resuspension or homogenization.of the cells is required, and the plasma membrane proteins are said to be stabilized by the addition of dimethylsulfoxide and FMA. Contamination with cytoplasmic organelles and membraneous material is also considered neglible, since these components remain attached to the flasks (Mc Clure *et al.*, 1979). Even though only half of the plasma membrane material is isolated with this procedure the yield of plasma membrane protein obtained is comparable with other methods.

1.8. Plasma Membrane Solubilisation Procedures

Plasma membrane proteins can be placed in three broad categories: (i) loosely-bound proteins which might be transiently located in the membrane, (ii) extrinsic or peripheral proteins that can be removed by conditions affecting the electrostatic environment of the

membrane, e.g. low ionic strength, and (iii) intrinsic or integral membrane proteins are tightly bound to the lipid, and extraction of these proteins requires the use of detergents that disrupt the hydrophobic interactions of the membrane.

Extraction procedures employed in the successful isolation of extrinsic and intrinsic membrane proteins will be discussed below. Loosely-bound proteins are readily washed away by physiological saline during preparation of the membrane, and are such not readily recognised as membrane proteins, even though they are essential for the complete functioning of the membrane (Neville, 1976). The solubilising effects of detergents stem from their ampiphilic character which enables them to interact with both hydrophobic and lipophilic regions of the proteins in an essentially disruptive fashion (Helenius and Simons, 1977). Two sets of interactions are considered significant in detergent-aided solubilisation of membrane proteins, the interaction of detergent molecules with membrane components and the interaction between detergent molecules. The binding of the detergent to the membrane and its protein is dependent on the number, and affinity of binding sites for the detergent molecules. Generally, sites of high affinity are few, and although these can be readily saturated at low detergent concentration, the binding frequently has a minimal effect on protein conformation. Detergent saturation of low affinity membrane proteins can be achieved by increasing the detergent concentration. However, increased detergent binding tends to affect the protein conformation. The interaction of detergent molecules with themselves is also important, since the concentration of detergent effective in binding is dependent on the concentration of free molecules of detergent (i.e., molecules not complexed into micelles) and not the total concentration of detergent itself. If the critical micellar concentration (CMC) of the detergent is sufficiently low, then the concentration of free detergent molecular will only be sufficient to saturate protein sites of high affinity for detergent, with minimal effect on protein conformation. Triton X-100 and sodium deoxycholate, both have low CMC values and, as such, are frequently used for the isolation of biologically active molecules.

Contrastingly, SDS has a high CMC, and as such, allows for high level binding and causes the complete disruption of the native conformation of the protein (Helenius *et al.*, 1979).

Non-ionic detergents are generally employed for the removal of integral membrane proteins, and peripheral membrane proteins which are aligned to the cytoplasmic side of the membrane. Water soluble proteins react mildly or not at all with non-ionic detergents. The process of solubilisation involves the substitution of hydrophobic lipids with the detergent. Since the membrane lipid and detergent may be regarded as competing with each other for hydrophobic regions in the membrane protein, the total mass of detergent used should be 10 times in excess of the mass of cell lipid. Under these conditions, thorough membrane solubilisation is said to occur (Bordier, 1981).

The protein detergent ratio that allows for maximum solubilisation of membraneous material is usually determined empirically for each particular system and membrane type. Generally, up to 5-10 mg of protein per 1 ml of detergent can be used. It has been found that most particulate fractions can be resuspended to this volume to mass ratio.
With the above concentration, the detergent to protein ratios (W/W) cover the range of 10:1 - 0.1:1. At a protein concentration of 1 mg/ml detergent concentrations between 0.01-1.0% should be used (Helenius and Simmons, 1977).

Electrostatic interactions are a well-known feature in intermolecular forces in all membranes. The type of buffer and ionic strength are two important parameters controlling membrane solubilisation. Buffers of high ionic strength, 0.1M - 0.5M dissociate membrane complexes held together by electrostatic forces. For example, phosphate buffers of 0.02M - 0.5M have been shown to be more efficient than Tris-based buffers. Phosphate buffers alone can solubilise many membrane proteins. This is thought to take place through the sequestering of divalent cations, and by the direct effect of phosphate on the structure of water. Polyols, like sucrose, glycerol, ethylene, and

propylene glycol are usually added to the membrane solubilisation buffers. They serve as protective agents and non-specific protease inhibitors at moderate concentrations (20% W/V). Peripheral membrane proteins are readily released into the soluble fraction, by any of the above-given treatments.

The ideal end point of solubilisation is the production of mixed micelles of lipids and detergent, and totally dilipidated proteins. The amount of membrane solubilised can be determined by evaluating the activity of a membrane marker protein, or direct assessment of the solubilised membranes by gel electrophoresis or gel filtration. If the detergent/protein ratio is too low, the detergent will only bind to membranes, but with an increase in the ratio successively smaller proteins will be generated into solution. However, residual protein - protein interactions may still be present at high detergent/protein ratios. If none, or low concentrations of protein activity are found in the detergent soluble fraction, using a non-ionic detergent only, then a mixture of non-ionic and ionic detergents is generally employed. Ionic detergents with a steroid nucleus, like sodium deoxycholate, digitonin octal glucoside or CHAPS are usually used.

1.9. **Aims of this Study.**

Human convalescent sera reacts with several infected cell polypeptides and plasma membrane proteins in both immunoprecipitation and immunoblot analysis (Sections 1.2.4. and 1.5). Monoclonal antibodies raised against infected cell polypeptides neutralise virus *in vitro*, and react with proteins on the surface of infected cells as shown by indirect immunofluorescence immunobinding assays (Pereira *et al*,1984).

Indeed, antibody and antibody dependent cell-mediated recognition of HCMV antigens on the infected cell surface have been implicated in playing a role in immune responses against HCMV (Middeldorp *et al.*, 1985, Sullivan-Tailyour, 1986; Stinski *et al.*, 1977). Human immune sera contains antibodies reactive with infected cell plasma membrane

proteins of 230-240,145-150, 98-103,78-80, 70-72,61-63, and 53-55 kDa (Sullivan-Tailyour and Garnett, 1986) and 34, 70-72, 98-103, and 145-150 kDa (Stinski *et al.*, 1979). Recently ^{125} I labelled exposed plasma membranes of HCMV infected fibroblasts of M.W. 53-63, 94, and 94-120 kDa were shown to react with human sera and monoclonal antibodies reactive with the virion gB glycoprotein complex (van der Voort *et al.*, 1989).

The aims of this study were to further characterise the immunogenic HCMV induced glycoproteins of M.W. 54, 62 and 94 kDa located in the plasma membranes of infected cells, and to determine the antigenic and structural association of these proteins to proteins of similar molecular weights found in the virion envelope. It had been previously shown that these proteins occured at high frequencies in FMA plasma membrane preparations, reacted with human convalescent sera and could be detected in high concentrations at 72h after infection (Table IV). These glycoproteins were also similar in molecular weights to some of glycoproteins reported in the virion envelope (Farrar and Oram, 1984), and glycoprotein complexes of either the gB or gCII complexes (Cranage *et al.*, 1986 ; Spaete *et al.*, 1989; Gretch *et al.*, 1988)

The occurence of the above plasma membrane proteins in the plasma membranes of infected cells was assessed by comparing proteins isolated from two different plasma membrane extraction and purification procedures, namely, the two-phase polymer system and the FMA extraction procedures (Section 1.7). Compositional changes in the plasma membrane proteins of uninfected and HCMV-infected fibroblasts were analysed by reducing SDS PAGE and image subtraction analysis of metabolically labelled protein profiles detected by SDS PAGE and fluorography.

Rabbit polyclonal sera raised against virus-induced plasma membrane proteins of M.W. 54 62, and 94 kDa, was used to determine the antigenic and structural relatedness of these proteins to those found in virions and virion envelopes. Similarly, monoclonal antibodies

and monospecific polyclonal antisera reactive with virion envelope proteins of the gB glycoprotein were used to determine the antigenic association between virion envelope proteins and virus-induced proteins in the plasma membranes of infected cells. Both non-reducing and reducing SDS gel electrophoresis were used to detect the possible formation of these proteins into high molecular weight complexes. The reduced sub-unit composition of the HMWC's were analysed by excising the HMWC's from the gels, reducing the proteins with 2ME and then re-electrophoresing these proteins under reducing SDS PAGE.

2. MATERIALS AND METHODS.

2.1. Cell Culture Techniques.

Eagles's minimal essential medium, heat inactivated foetal calf serum trysin and glutamine were purchased from Flow, USA; GIBCO, U.K; CSL, Aust. Tissue culture flasks, 24 well tissue culture trays, 96 well microtitre plates and cell scrapers were purchsed from Flow, USA and Costar, USA. Penicillin, streptomycin and 4-6-diamine-2-phenylindole dihydrochoride were purchased from Boehringer Mannheim, W. Germany.

2.1.1. Cell Culture.

During the course of the project human fibroblasts from the following sources were used.

(1) Human embryonic lung fibroblasts (HEF) were obtained from foetal lungs. The method of Hayflick and Moorhead (1961) was used to establish primary HEF cultures *in vitro*. Foetal lung tissue was asceptically removed, placed into Hanks Balanced Salt Solution (HBSS) pH 7.2 (Appendix), cut into small fragments, and treated with trypsin:ethylenediaminetetra-acetic acid (EDTA) solution
(0.25% trypsin (w.v), 0.05% EDTA (w/v) in calcium and magnesium free phosphate buffered saline (PBS) at pH of 7.2-7.4 (Appendix). This mixture was placed on a shaker at 37^{o}C, and the supernatant containing single cells decanted every 10 min. Fresh trypsin EDTA solution was replaced and the above procedure repeated at least 4 times. Cells were pelleted at 1,500 rpm for 10 min in a Beckman TJ6 bench top centrifuge resuspended in growth medium and seeded at a density of 5 x 10^7 cells/ml into 25 cm^2 tissue culture flasks. The growth medium used was Eagle's minimal essential medium (MEM), with Hanks salts (Appendix), containing non-essential amino acids, 0.42g/l sodium bicarbonate 2,923 mg/l glutamine, 100 µg/ml penicillin, 100 µg/ml streptomycin and supplemented with 10 per cent (%) heat inactivated foetal calf serum (FCS) for

growth, and 2% FCS for maintenance. The pH of the culture medium was maintained at approximately 7 by the addition of a 7.5% sodium bicarbonate (w/v) solution.

After 12 h of cell culture, all non-adherent cells were removed by decanting the growth medium. The adherent cells were grown to confluency, with replacement of the growth medium every second day. Confluent monolayers of HEF were then subcultured by treatment of the monolayer with trypsin: EDTA (0.25% (w/v): 0.05% (w/v)) for 5-10 min at 37°C. Cells were collected by centrifugation at 1000 rpm for 10 min, resuspended in growth medium and three flasks seeded from the yield of one.

Fibroblasts were used between the fifth and twentieth passage, for experimental work. HEF's were also grown as monolayers on 10mm glass coverslips (Chance, U.K.) in 24-well trays or in 96-well flat bottomed microtitre plates. Eagle's MEM with Earle's salts containing non-essential amino acids, 2.2g/l sodium bicarbonate, 2.923 mg/l glutamine, 100 µg/ml penicillin, 100 µg/ml streptomycin, supplemented with 10% FCS was used to culture cells in microtitre plates. Cultures were incubated in a carbon dioxide incubator at 37°C containing 3-5% CO_2.

(2) Human foreskin fibroblasts (HFF) were obtained at passage 4 from Westmead Hospital (Sydney, Australia), and used between the 6th and 15th passages. They were routinely cultured in MEM containing Hanks Salts as described above.

2.1.2. **Cell Stocks.**

Cells were stored at -70°C in medium consisting of either 75% MEM, 15% FCS and 10% glycerol (v/v) or 10% dimethylsulphoxide (Me_2SO_4) 15% FCS and 75% MEM. After trypsinization, cells were suspended at a concentration of 5×10^7 cells/ml, decanted in 1 ml aliquots into Nunc tubes, and frozen slowly to -70°C. When required, the cells were thawed rapidly at 37°C, diluted with MEM and pelleted. Cells from each vial were

routinely resuspended in 8ml fresh growth medium and seeded into 25 cm^2 tissue culture flasks to allow for adherence and growth of viable cells. After 12 h cells that had not attached were decanted and the monolayer washed twice with HBSS or PBS pH 7.4 before replacing fresh growth medium.

Cell stocks and monolayers of HFFs and HEFs were routinely screened for the absence of mycoplasmas using 4-6-diamine-2-phenylindole dihydrochloride (DAPI) (Appendix).

2.2. Virus.

2.2.1. Virus stocks.

Human cytomegalovirus (HCMV) strain AD169, supplied by American Type Culture Collection, Rockville, Maryland, was serially passaged to yield a high titre stock. Stock virus was obtained by infecting sub-confluent HEF or HFF monolayers (approximately 10^5 cells per cm^2) in 150 cm^2 flasks with MEM containing approximately 2 x 10^7 virus particles (multiplicity of infection (m.o.i.) of two plaque forming units (PFU) per cell). The cells were washed (2x10 min) with HBSS to remove any serum components, which prevent virus binding. After an adsorption period of 90 min at 37°C, the inoculum was replaced with maintenance medium. The medium was harvested every 48 h and replaced with fresh maintenance medium. At approximately 10 days after infection when over 80% of the cells showed advanced cytopathic effects (CPE), the medium and infected cells were harvested. An equal volume of 70% sorbitol (w/v) was added to the flasks, and the infected cells removed by scraping with a cell scraper , and the stock stored at -70°C. Before use, the virus stock was freeze-thawed twice to facilitate cell lysis and release of cell associated virus.

2.2.2. Virus Titrations.

A tissue culture infectivity assay based on the methods given by Wentworth and French (1970) was used to determine the approximate titre of the virus stock. Subconfluent monolayers of HEF grown in 96-well microtitre trays were inoculated with serial dilutions of 10^{-1} through to 10^{-10} of stock virus. Virus was adsorbed for 90 min, decanted and replaced with maintenance medium. Infected cells were incubated at 37°C. Maintenance medium was replaced every 48 h up until 7 days after infection. Cells were washed twice with PBS pH 7.4, before fixing and staining with Quick Dip I and II (Histo-Labs, NSW). The infectious dose fifty (ID_{50}) was taken as the highest dilution which gave an observable cytopathic effect in 50% of the wells. The virus titre (PFU/ml) was estimated from the ID_{50}. The titre of the virus used to infect subconfluent monolayers of HEFs for isolation of proteins and plasma membranes was 10^{7}-10^{9} PFU/ml. Monolayers of 90% confluent HEF were inoculated with virus at a m.o.i. of 5-10 PFU per cell.

2.2.3. **Virus Isolations.**

(i) Extracellular and intracellular virus.

At 7 days post infection, HCMV infected monolayers were scraped off into the tissue culture medium, freeze-thawed 3 times to allow for efficient release of virus and centrifuged at 1,000 rpm for 5 min in a Beckman, TJ6 centrifuge to remove cell debris, and then at 7,000 rpm for 20 min to remove any particulate material. The medium was subsequently centrifuged at 20,000 rpm for 60 min in a Beckman SW 41 rotor to pellet enveloped virions and dense bodies. The pellets were pooled and resuspended in a total of 4 ml of TN buffer (0.05M Tris/HCl; 0.1M NaCl) pH 7.4.

(ii) Extracellular virus.

Supernatant fluid was collected from HCMV infected monolayers of HEF, and clarified by centrifugation at 2000 rpm for 10 min at 4^0C to remove any cellular debri. The virus containing fluid was then either centrifuged at 40,000 rpm for 2h to pellet the virions (Gupta, *et al.*, 1977) or concentrated with polyethylene glycol.

Polyethylene glycol (PEG) 6,000 (5% v/v) was added slowly to the supernatant fluid (pH 7.0). The salt concentration and pH were adjusted before precipitation using solid NaCl, and HCl (6N) or NaOH (5N) solutions to 0.15M and pH 7.0, respectively. This suspension was kept at 4^0C with stirring for 4 h, and the sedimentable phase collected by low speed centrifugation, at 3,000 rpm. The virus pellet was resuspended in 10 ml (1/100 of the initial volume) of TN buffer pH 7.4

(iii) Intracellular virus.

To isolate intracellular virus particles from infected cells, HEF monolayers in 175 cm^2 tissue culture flasks showing advanced CPE were washed with 15 ml of PBS pH 7.2 (3 x 10 min each). Cells were scraped into 10 ml of pH TN buffer, frozen and thawed three times and disrupted using a 20 ml glass homogeniser with 40 strokes of a tight fitting pestle. The homogenates were centrifuged in a Sorvall SS34 rotor at 10,000 rpm for 20 min, and the intracellular virions purified from the supernatant fluids by glycerol tartrate centrifugation as described in Section 2.2.4.

2.2.4. **Virus Purification.**

Isolated intracellular and extracellular virus or a mixture of both, were resuspended in TN buffer pH 7.4 (approximately 4ml per isolation) and used for further purification. During the course of this work, three methods of virus purification were employed, with the main objective being to achieve high yields of undamaged virus.

(i) Discontinuos sucrose gradient centrifugation.

The method of Gupta, *et al.*, (1977) was followed. Virus suspensions were treated with 100mg/ml DNAse at 37 C for 30 min., and centrifuged at 4,000rpm to remove any cell debri that had aggregated to the virus. The supernatant fluid was then applied to discontinuos sucrose gradients of 60%, 45% and 22% (w/v) in PBS, pH 7.4. The gradients were centrifuged at 38 000 rpm for 2h. at 4 C in a SW 41 rotor (Beckman). Enveloped virions babned at the 22% - 45% sucrose interface, and dense bodies at the 45% - 60% interface. Virion and dense body fractions were then dialysed against PBS to remove any remaining sucrose.

(ii) 20%-70% (wt/vol) linear D-sorbitol gradient centrifugation.

The procedure of Stinski (1976) was followed. Virus suspensions were treated with 100mg bactracin/ml and 0.5M urease to prevent aggregation of virions and dense bodies. The virus suspension was sonicated for 15 sec. and layered onto 20 - 70% (w/v) linear D - sorbitol gradients in Tris buffered saline (TBS), pH 7.4. The gradient was centrifuged at 22,500 rpm for 1 h. in a SW 25 rotor (Beckman). Virions and dense bodies which banded in the region of 55% D- sorbitol, were collected, diluted with 2 vol TBS, pH 7.4, and dialysed against TBS.

(iii) Glycerol potassium tartrate gradient centifugation.

The procedure of Talbot and Almeida (1977) was followed. Virus suspensions (4 ml) were layered onto gradients of 30% glycerol in TN buffer (w/w) containing 15% potassium tartrate (w/w) and 35% potassium tartrate in TN buffer (w/w) and centrifuged for 15 min at 40,000 rpm in a SW 41 rotor (Beckman).

Under these conditions, enveloped virions and dense bodies sedimented to form two distinct well-separated light scattering bands. Both the upper band containing the

enveloped virions and the lower band containing the dense bodies were separately aspirated, diluted with 3 ml TN buffer and recentrifuged under identical conditions using fresh gradients. This was repeated twice and the final fractions processed for either SDS PAGE analysis (Section 2.8.) or the enveloped virion fraction treated with detergents (Section 2.3.2.).

2.3. Plasma Membrane and Virion Envelope Isolations.

2.3.1 Plasma membrane isolations from uninfected and HCMV infected fibroblasts.

Three methods of plasma membrane isolation were employed during the course of this study.

(i) Plasma membranes were isolated by a modification of the fluorescein mercuric acetate technique of Sher and Barland (1972) as given by Sullivan-Tailyour (1986). This method was used primarily to characterise virus-induced plasma membrane proteins. HCMV infected and uninfected monolayers of fibroblasts in 175 cm^2 tissue culture flasks were washed three times with 15 ml of 0.16M sodium chloride (NaCl) containing 0.1M calcium chloride ($CaCl_2$) at room temperature (RT). The cells were then treated for 10 min with 1mM zinc chloride ($ZnCl_2$) in dimethylsulfoxide (Me_2SO_4) (4:1 ; v/v) at RT. This solution was decanted and 20 ml of cold saturated fluorescein mercuric acetate (FMA) (approximately 2.2 x 10^{-3}M) in 0.02M trishydroxyaminomethane-hydrochloric acid (Tris-HCl) at pH 8.1 was added. The cells were placed on ice and shaken for 45 to 50 min on a rotary shaker set at 120 rev/min. Material released into the FMA/Tris solution was pelleted at 600 x g for 10 min and the resulting pellet washed with 20 ml of 1mM sodium bicarbonate ($NaHCO_3$) to ensure removal of excess unbound FMA from the membrane fragments. This procedure was repeated 2 to 3 times, and the purity of the membrane fragments assessed visually by

phase contrast and light field microscopy. The protein concentration was estimated to be approximately 60 μg-80μg per 175 cm^2 flask, as determined by methods in Section 2.3.3.

The final membrane pellet was resuspended in either 80 μl of SDS PAGE sample buffer (Appendix) for electrophoretic analysis or 100 μl of immunoprecipitation buffer (Appendix) as part preparation for determining the reactivity of membrane proteins with antisera.

(ii) An alternative method of plasma membrane extraction involving cell fractionation and two-phase polymer extraction procedures was also carried out. Modifications of the procedures given by Brunett and Till (1971), Klockman and Deppert (1983a) and Rana and Majumber (1987) were used.

Monolayers of fibroblasts (175 cm^2 tissue culture flasks) were washed three times for 5 min each with 15 ml KM buffer (see Appendix), pH 6.2. Cells were scraped into 10 ml Lysis Buffer (Appendix), pH 6.5, incubated for 15 min at room temperature, cooled on ice for 5 min, and then ruptured in a 40 ml Dounce homogeniser with a tight-fitting (Type B) pestle (Wheaton Scientific, USA). The homogenisation, as well as the subsequent steps in the procedure, were monitored by phase-contrast microscopy.

Twenty to twenty-five strokes were usually required for uninfected cells between passage 10 and 20 and 12-15 strokes for infected cells. The degree of cell confluency greatly affected the number of strokes required to rupture the cells. Uninfected cells were routinely used one to two days after passage. Homogenisation was stopped when greater than 90% of the cells were ruptured. The homogenate was placed into 30 ml Corex-glass tubes (Sorvall Instruments, Du Pont), and centrifuged for 15 min at 1400 rpm in a Sorvall HB4 rotor. The supernatent was discarded and the pellet resuspended in 10 ml of the top phase solution (Appendix), and then mixed with 10 ml of the bottom phase solution

(Appendix). The two phases and pellet were mixed by repeated inversion, and centrifuged at 8500 rpm for 10 min in a Sorvall HB4 rotor. The pellet was discarded and the membranes found at the interface of the two phase system remixed and recentrifuged at 8500 rpm for 10 min. The supernatant and membrane interface fraction was similarly remixed and recentrifuged. Plasma membranes were harvested from the interface and diluted 4 x vol with gd water. The membranes were pelleted by centrifugation at 2,000 rpm for 15 min. This step was repeated twice to ensure thorough removal of any dextran and PEG. The protein concentration of the membrane-enriched fraction was estimated to be approximately 40 μg - 60 μg per 175 cm^2 flask as determined by methods given in Section 2.3.3. The pellets were resuspended in either 100 μl of immunoprecipitation buffer (Appendix) or directly into 50 μl SDS sample buffer (Appendix).

(iii) An alternative method for isolating plasma membrane, based on the procedure given by Krah and Crowell (1982, 1985) was used to prepare membranes for immunoabsorption chromatography.

Monolayers of fibroblasts (175 cm^2 tissue culture flasks) were washed three times with 15 ml PBS, pH 7.4 and then incubated with 20 ml of 2mM EDTA for 10 min at 37^0C to disrupt the monolayer. Cells were pelleted by centrifugation at 1,000 rpm for 10 mins in a Beckman TJ6 bench top centrifuge, and washed by centrifugation in 10 ml of 50mM Tris-HC1, pH 7.4 at 1,000 rpm for 10 min. The pellet was resuspended in 5 ml 0.2M hypotonic phosphate buffer, pH 7.0 and allowed to swell at 4^0C for 30 min. Cells were then ruptured by homogenisation with a 10 ml Dounce homogeniser with a tight fitting pestle (type B). Approximately 30 strokes were used. The homogenate was then placed through three cycles of rapid freezing to -70^0C and slow thawing. EDTA (0.1%) containing 1% glucose was added to disaggregate membrane clumps and the homogenate centrifuged at 1,000 rpm for 10 min. The supernatent was then centrifuged at 2,200 rpm for 15 min in a HB4 rotar (Sorvall) to remove mitochondria and the resulting supernatant containing membrane fragments centrifuged at 30,000 rpm in a Beckman SW 40 rotor to

pellet the membrane fraction. The membrane pellet was washed twice by centrifugation at 30,000 rpm with calcium and magnesium free PBS pH 7.2%. The final fraction was resuspended in 0.4% sodium deoxycholate and treated with ultrasound (two bursts of 30 sec) in order to further solubilise the membrane fragments. After an incubation period of 30 min at 4^0C, solubilised membranes were dialysed against five changes of PBS to remove excess detergent and then concentrated down to a volume of 2 ml (from 4-5 175 cm^2 tissue culture flasks) in a Minicon (Amicon, MA, USA). The protein concentration was determined before loading onto immunoaffinity columns. Approximately 40 µg of protein per 175 cm^2 flask of confluent fibroblasts was detected in the final plasma membrane fraction.

2.3.2. **Virion Envelope Isolations.**

Fractionation of enveloped virions by osmotic shock and with detergents was carried out according to the procedures given by Farrar and Oram (1984). Fragments of virus envelope were obtained by osmotic shock treatment of enveloped virions. Concentrated virus suspensions in TN buffer were diluted with 50% vol water at 0^0C for 30 min, followed by centrifugation through 9 ml glycerol potassium tartrate gradients (Section 2.2.4.) in a Beckman SW 28 rotor at 27,000 rpm for 20 mins. Bands containing either virions or envelope fragments were removed by aspiration, diluted with 2 vol Tris buffered saline (TBS), pH 7.4, and used either directly for assessment by scanning electron microscopy or recovered by centrifugation in a Beckman SW 28 rotor at 27,000 rpm for 30 min.

Virions recovered after osmotic shock treatment were resuspended in TN buffer containing 1% Triton X-100, or 1% sodium deoxycholate, and subjected to ultrasound (2 bursts of 30 sec at 0^0C) in an ultrasonic waterbath (Bransonic B12; 50/60 Hertz/80 Watts) and then incubated for 20 min at 0^0C. The dispersed virus suspension was centrifuged at 21,000 rpm for 40 min at 0^0C in a Beckman 55 Ti rotor. The supernatant,

containing the solubilised envelope fraction was collected. Triton X-100 solubilised envelopes were then treated with SM-Bio beads (Biorad,USA) (Appendix) to remove excess detergent prior to dialysis of both Triton X-100 and sodium deoxycholate solubilised envelope proteins fractions against 5,000 vol 0.1M acetic acid for 24 h at 4^0C. Solubilised envelope proteins were then concentrated by freeze drying and resuspended in 50 µl of PBS pH 7.2

2.3.3. **Protein Estimations.**

The protein concentration of all plasma membranes irrespective of the preparations, were estimated using Coomassie Brilliant Blue (CBB) G-250 (Bradford, 1976) and the Bio-Rad protein assay system (Appendix). The pH of the sodium bicarbonate was adjusted to pH 7.0 - 7.1 as a high pH interferes with colour development. The Bio-Rad protein assay system is based on the shift in the absorbance maximum of CBB G-250 from 465nm to 595nm when binding to protein occurs (Bradford, 1976). In all cases a standard curve of absorbance versus concentration of protein was drawn up for bovine serum albumin (BSA) in the concentration rage of 0 -1 mg/ml protein and the unknown protein concentrations were read off the curve.

2.3.4. **Evaluation of Plasma Membrane Purity.**

Since fluorescein mercuric acetate inhibits enzyme activity, an enzyme assay to assess the purity of isolated plasma membranes was carried out for membranes isolated by the two phase polymer extraction procedures only.

Na^+, K^+-ATPase was used as an enzyme marker for plasma membranes and assayed according to the method given by Esmann (1988). Na^+, K^+-ATPase activity is defined as the ouabain sensitive hydrolysis of ATP in the presence of Na^+, K^+ and Mg^{+2}. Since 1mM ouabain in the Na^+, K^+-ATPase reaction mixture has been shown to completely

inhibit Na^+, K^+-ATPase activity in most tissues, the ATPase assay was performed in the presence and absence of ouabain. As such a mixture of ouabain and membranes serves as a blank for non-specific ATPase activity as well as a reagent blank. The substrate consisted of 100 µl 1.3M NaCl; 100 µl 0.2M KCl; 100 µl 30mM $MgCl_2$; 100 µl of plasma membranes (50 µg) or cell homogenate (50 µg). Ouabain (200 µl of a 100mM solution) and 200 µl of gd water were added to the reagent blank and 300 µl of gd water to the enzyme assay.

The reaction was allowed to proceed for 10 min at 37°C, terminated by the addition of 100 µl of 50% TCA, and cooled to 4°C. Protein was removed by centrifugation at 10,000 rpm for 10 min at 4°C in a Sorvall SS34 rotor. The clarified supernatant (500 µl) was used to determine the amount of phosphate liberated from the membrane sample. The following reagents were added on ice, the membrane sample or cell homogenate (500 µl), 1.5µl copper sulphate, pH 4.0, and 250 µl ammonium molybdate. The mixture was vortexed and then 250 µl p-methylaminophenol sulphate (ELON) was added and vortexed again. The mixture was incubated at 25°C for 7 min, and then placed back on ice. Samples were read spectrophotometrically at 750nm, and the amount of phosphate determined by reference to a standard curve.

2.3.5. **Assessment of Virion Envelope Purity.**

Purified virus suspensions (5 ml) or virion envelopes solubilised with Triton-X-100 (5 ml) were placed on dental wax, and formvar coated grids placed on top of the drops in an inverted position, and left for 90 min for the virus and/or envelopes to adhere. The grids were then washed thoroughly with gd water, dried and placed on drops (5 ml) of 0.1% uranyl acetate in ethanol:water (1:1, v/v) (adjusted to neutrality with 1N NaOH) for 30 sec, washed, dried and viewed on a Cambridge S.4 scanning electron microscope.

2.4. **Solubilisation Techniques.**

2.4.1. **Plasma Membranes.**

Several methods of protein solubilisation were undertaken during the course of this study these include:

(i) Direct solubilisation of plasma membranes and virions with reducing SDS PAGE Sample Buffer.

Plasma membrane fragments obtained from FMA or 2-phase polymer extraction from HEF's or HFF's were directly solubilised in SDS PAGE sample buffer (Appendix) containing 2% sodium dodecyl sulphate (SDS) (w/v) and 2% β mercaptoethanol (2-ME) (v/v). Membrane pellets obtained from a 175 cm^2 monolayer of fibroblasts (Section 2.3.1) were resuspended in 50 µl - 80 µl of SDS PAGE sample buffer and thoroughly vortexed. Samples were heated at 100^0C for 3-5 min to complete the protein disaggregation, centrifuged at 1,000 rpm for 60 sec in a Beckman Microfuge to remove any particulate material, and loaded directly onto SDS PAGE reducing gels.

(ii) Direct solubilisation of plasma membranes with non-reducing SDS PAGE sample buffer.

Plasma membrane samples were solubilised directly in SDS PAGE sample buffer (Appendix) containing no 2-ME. Solubilisation was carried out for 30 min at RT with mixing, centrifuged for 30 sec at 1,000 rpm to remove any unsolubilised particulate material and loaded directly onto gels. Samples were not subjected to 100^0C for 3-5 min.

(iii) Solubilisation with 1% sodium deoxycholate and 1% NP40.

Plasma membranes isolated from fibroblasts by FMA extraction and two-phase polymer systems were treated with RIPA buffer (Appendix) containing 1% sodium deoxycholate and 1% NP40 for 30 min of ice with occasional mixing. These samples were routinely used for immunoprecipitation analysis (Section 2.1.2).

2.4.2. **Whole Cell Solubilisation.**

HCMV infected and unifected HFF's were scraped directly from the monolayer, and treated with RIPA buffer (Appendix) for 30 min. at 4 C. Cells that had been treated with FMA, after the removal of the upper plasma membranes were as the above cells. The suspended cells were then homogenized (30 strokes) in a Dounce homogenizer with a tight fitting, type B pestle. These suspensions were centrifuged at 30,000rpm in a 75Ti rotor (Beckman), the supernatants collected and used for immunoprecipitation analysis. (Section 2.12).

2.4.3. **Virion Solubilisation.**

Isolated virions and dense bodies (Section 2.2.4) and virion envelopes obtained either from osmotic shock or detergent treatment (Section 2.3.2.) were treated with 2 vol reducing SDS PAGE sample buffer. The samples were heated for 3 min. at 100 ^{o}C, before application to reducing SDS gels.

2.5 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE).

(1) Reducing Gel Electrophoresis.

Plasma membrane proteins, whole cell protein lysates, virions, virion envelopes and immunoprecipitates prepared by methods given in Section 2.4., were separated according

a modification of the procedure described by Laemmli (1970). Linear slab gels used during the course of this work were of 5%, 7.5%, 10% or 12% acrylamide for the resolving gel and a 4% stacking gel (Appendix). Gradient slab gels consisted of 10%-20% or 7.5%-15% acrylamide (Appendix). The size and thickness of the gels varied according to the type of apparatus used and the amount of protein loaded onto the gels. Gels of 100 mm x 80 mm x 3 mm or 1 mm were run in a Pharmacia vertical slab gel apparatus (Pharmacia, Sweden). Gels of 150 mm x 160 mm x 1.5 mm were run in a Bio-Rad Protean II Apparatus (Biorad,USA), and gels of 150 mm x 120 mm x 1.5 mm run in electrophoretic tanks built according to the specifications of Andrews, (1986). The gels were pre-run before use and electrophoretic separation was carried out in a continuous tris-glycine buffer system at a pH of 8.5 (Appendix). Electrophoresis was performed at room temperature at 35-40 mA per gel. The tracking dye used to monitor the run was 0.2% bromophenol blue in tris-HCl pH 6.8 (w/v) (Appendix).

The gels were fixed in 10% to 12% trichloroacetic acid (TCA) (w/v) for 3 h or overnight, or placed directly into 0.1% CBB-G250 (LKB) dissolved in water. Alternatively, proteins were stained with either 0.25% CBB R-250 (w/v) (LKB) dissolved in methanol-acetic acid-water (45:10:45 by volume), or with silver nitrate. CBB R-250 stained gels were destained electrophoretically in the above solvent for 1.5 to 2 hr at 12V in a Pharmacia destaining apparatus. Gels were stained with silver nitrate according to the Bio-Rad Silver stain kit specifications or the procedure given by Marshall *et al.*,(1981 ; Appendix).

Stained gels were recorded (i) photographically using a CU5 polaroid hand camera or a Polaroid MP-4 land camera and (ii) densitometrically using an enhanced laser densitometer (Ultroscan XL 2222-020, LKB).

The molecular weights of the membrane proteins were calculated from the migration of standard proteins of known molecular weight. Pharmacia high and low molecular weight

calibration kits were used (Appendix). Pre-stained Bio-Rad molecular weight markers were used to monitor the electrophoretic separation
(Appendix). The position of each band was measured after staining and a standard plot of the logarithm of molecular weight versus relative mobility was drawn (Shapiro, Vinuela and Maizel, 1967; Weber and Osborn, 1969). The molecular weights of all proteins are given as the mean in a range of 2 kilodaltons.

2.6 Antisera.

2.6.1 Antisera Production

(i) Plasma membrane proteins.

Stained gel bands of glycoproteins of 54 62 and 94 kDa obtained from FMA infected cell membrane extracts fractionated by SDS PAGE were excised from 10% gels and successively passed through needles of the following diameters: 1.20mm, 0.8mm and 0.6mm. The macerated gel fragments (1 ml) were mixed with an equal volume of Freund's incomplete adjuvant (Difco,USA) and further homogenised in a 10 ml glass homogeniser with a tight fitting pestle. Four bands containing approximately 20 mg of protein were inoculated intramuscularly at weekly intervals into New Zealand white rabbits. Four inoculations were given and a final booster in the fifth week. The γ globulins of collected sera were precipitated with saturated ammonium sulphate and dialysed against 5-volume changes of borate buffer, pH 7.4. The IgG fraction was purified using a protein A Sepharose CL 4B affinity column (Section 2.6.1).

HCMV enveloped virions purified by glycerol potassium tartrate gradient centrifugation (Section 2.2.4) were inoculated into New Zealand white rabbits. The immunisation schedule, given above was followed except that 1 vol (1 ml) of virus suspension in PBS was mixed with an equal volume of Freund's complete adjuvant (Difco,U.K.).

(ii) IgG Preparation.

Saturated ammonium sulphate (pH 7.2) was added dropwise to rabbit serum to a final volume of 1:2 stirred for 20 min at 25°C, and centrifuged at 200 rpm for 30 min to sediment the precipitable fraction. The γ globulin fraction was resuspended in 0.1M borate buffer pH 7.4 (Appendix), and dialysed against five changes of borate saline buffer pH 8.3-8.5 (Appendix) at 4°C. After dialysis, the protein concentration of the γ globulins was determined using the Bio-rad protein assay system (Section 2.3.3). If further concentration of the γ globulin fraction was required the dialysis tubing was placed directly into PEG 6000, to allow for absorption of excess water, and then re-dialysed as above.

The γ globulin fraction (1 mg protein/ml) was passed through a glass wool column with an equal volume of buffer A (Appendix) to ensure the removal of any particulate matter before application of this sample onto the protein A column (Appendix). Buffer A (5 to 10 volumes of the void volume of the column) was added to the column in order to elute the IgM fraction. The flow rate was then adjusted to 1 drop per 15 sec and the IgG fractions collected by elution with buffer B (Appendix). IgG fractions were read spectrophotometrically at an absorbance of 280 nm until the absorbance was near zero. IgG fractions were pooled and dialysed against gd H_2O at 4°C, concentrated with PEG 6000 and re-dialysed against five changes of PBS buffer pH 7.4 at 4°C. The protein concentration of the IgG was adjusted to 1 mg/ml. For long-term storage over six weeks, IgG fractions were kept at -20°C, and for shorter time periods at 4°C with 15mM sodium azide.

2.6.2. Antisera used for Immunoprecipitation Analysis.

The following antisera preparations were used for immunoprecipitation analysis (Section 2.12) with :

(1) Uninfected and HCMV infected plasma membranes proteins and whole cell protein lysates.

(i) Protein A sepharose purified IgG from rabbit sera raised against infected plasma membrane glycoproteins of M.W. 54, 62 and 94 kDa.

(ii) Complement dependent neutralising murine monoclonal antibody 15D8, reactive with the gp 55 of HCMV glycoprotein complex gp 55-130 (gB) of the virion envelope (Donated by L. Rasmussen, Division of Infectious Diseases, Stanford Medical School, Stanford, California).

(iii) Neutralising murine monoclonal antibody 7-17 reactive with glycoprotein complex gp55-116. (Donated by W. Britt, Virology Laboratory, Department of Pediatrics and Microbiology, University of Alabama at Birmingham, Alabama).

(iv) Monospecific polyclonal guinea pig antisera reactive with HCMV envelope proteins and infected cell proteins gp 55 and 130. Serum 6PP and 4PP were donated by E. Gonczol, Wistar Institute, Philadelphia.

(v) Murine monoclonal antibody CH28-2, reactive with the gA complex of glycoproteins in HCMV infected cells. (Donated by L. Pereira, Viral and Rickettsial Disease Laboratory, California Department of Health Service, Berkeley, California).

(2) Virion proteins or solubilised virion envelope proteins.

(i) Anti-54 kDa IgG, anti-64 kDa IgG and anti-94kDa IgG (see Section 2.5.1)

(ii) Immune serum from HCMV-positive patients. (Donated by J. Alexander, Department of Microbiology, University of the Witwatersrand, Johannesburg, South Africa).

(iii) Rabbit antisera raised against virions (Section 2.6.1).

(vi) Monoclonal antibody α 52, reactive with the gp 52 of the gB complex of glycoproteins in the virion envelope. (Donated by J. Oram, Public Health Laboratory Services, Porton Down, Salisbury, England).

2.7. **Antibody Binding Assays: Immunoperoxidase Staining.**

Subconfluent fibroblasts grown on glass coverslips were infected with HCMV (Section 2.1.1) and fixed at 80 hrs post infection with 2% freshly prepared paraformaldehyde in PBS pH 7.4 for 20 min at 4°C. Fixed cells were washed with PBS and treated with a 1/50 dilution of HCMV negative human sera to block any non-specific binding of antisera to fibroblast F_c receptors, induced by HCMV infection. Cells were routinely washed (three times for 10 min) with PBS between antisera treatments. Antisera raised against the 54, 62 and 94 kDa plasma membrane proteins and appropriate dilutions of these sera were added to the cells for 1 h at RT. The second antibody, goat anti-rabbit IgG conjugated with horse radish peroxidase (Cappel, USA) at a dilution of 1:10,000 was added to the cells and was incubated for 1 hr at RT. The chromagen 3,3' diaminobenzidine tetrahydrochloride (DAB) (Sigma,.USA) (0.2%) and 0.001% hydrogen peroxidase was added to 10 ml of distilled water, and the cells incubated at RT until colour development. The coverslips were washed extensively with PBS pH 7.4 and mounted onto slides without prior dehydration in 9 parts glycerol:1 part PBS. Slides were viewed under a Leitz Dialux 20 microscope using bright-field illumination for visualisation of brown deposits indicating immunoperoxidase staining.

The following procedures were performed as controls

(i) infected cells were reacted with peroxidase conjugate and DAB.

(ii) uninfected cells were reacted with conjugate and DAB.

(iii) infected and uninfected cells were reacted with PBS and DAB.

(vi) infected cells were reacted with pre-immune rabbit sera, goat anti-rabbit IgG and DAB.

(v) uninfected cells were reacted with anti-54, 62 and 94 kDa sera.

2.8. Virus Neutralisation.

Microneutralisation assays were performed by titrating extracellular virus in the presence and absence of Protein A purified IgG from rabbit serum raised against plasma membrane proteins 54 and 62 kDa isolated from HCMV infected fibroblasts. Anti-54 kDa IgG was used at a concentration of 20 μg of protein and anti- 62 kDa IgG at a concentration of 25 μg of protein . Stock virus at a titre of 10^9 was used at dilutions of 10^{-1} through to 10^{-9}. Fresh reconstituted guinea pig complement (Cappel, USA) was used at a concentration of 2% in PBS pH 7.2. Subconfluent monolayers of HEF in flat bottomed 96-well microtitre plates (Section 2.1.1) were washed three times for 10 min intervals with PBS, pH 7.4 . Mixtures of virus and anti-54 kDa IgG or anti-62 kDa IgG with and without complement were pre-incubated at 37^0C for 45 min before addition to the cells . The cells were then incubated at 37^oC for 60 min to allow for the adsorption of free virus. This mixture was decanted and replaced with MEM supplemented with 2% FCS. The cells were incubated for six days, with replacement of fresh maintenance medium every second day. The development of CPE in the cells to which only the virus had been added was monitored visually using inverted microscopy. Monolayers were washed three times with PBS and fixed and stained with Quick Dip I and II (Histolabs, Aust.) and the number of CPE scored for all wells. Antisera were considered neutralising if the number of CPE scored was less than 50% of that of the control wells to which an equivalent concentration of virus without antisera had to be added.

2.9. Immunoblotting Techniques.

(i) Protein transfer.

Electrophoretic transfer of proteins separated on SDS-polyacrylamide gels was carried out according to the procedure of Burnette (1981) adapted from
Towbin *et al..*, (1979). SDS PAGE gels were washed in two changes of distilled water to remove any excess SDS on the surface of the gels, and then with 2 changes of transfer buffer (Appendix). Nitrocellulose (0.45 mm pore size, Zeiss) Whatman's number 1 filter paper and Scotch-Brite sheets were all pre-soaked in transfer buffer. A sandwich of the following successive layers was prepared: (i) Scotch-Brite sheet; (ii) two sheets of filter paper; (iii) two nitrocellulose sheets cut to the size of the gel; (vi) SDS polyacrylamide gel; (v) two sheets of filter paper; (vi) one Scotch-Brite sheet. The assembly was secured with rubber bands placed in a gauze basket in a Pharmacia destaining apparatus (Pharmacia, Sweden) with the nitrocellulose toward the anode, and filled with transfer buffer. Electrophoretic transfer was accomplished at 1-12A (24-36V) for 4-6 hrs with buffer recirculation.

For direct visualisation of proteins after completion of the transfer, the nitrocellulose was washed three times in gd water and placed into a solution of 0.1% Amido Black in methanol:acetic acid: gd water (45:7:48; v:v:v) for 5 min, and subsequently destained in methanol : acetic acid gd water (70:7:23 ; v:v:v). Molecular weight standards were routinely stained and used in conjunction with the immunoblots to determine the molecular weights of proteins reactive with antisera.

(ii) Immunoblotting.

Dried nitrocellulose sheets were placed in Tween Tris buffered saline (TTBS) (Appendix) for 15-20 min on a rotary shaker 60 rpm; and then transferred to blocking solution containing 3% BSA or casein (Appendix) for 45 min after which they were washed twice in TTBS for 5 min each. The blots were then transferred to antisera buffer (Appendix) containing 1:100 dilutions of antisera and incubated for a further 45 min. The nitrocellulose strips were then washed with TTBS for a 10 min interval and then two successive 5 min intervals before transferring to antisera buffer containing goat anti rabbit IgG conjugated to horse radish peroxidase (GAR-HRP) at a 1:3000 dilution, and incubated for a further 60 min.

The immunoblots were then washed for 1 x 15 min and 2 x 15 min periods in TTBS followed by 1 x 5 min in TBS. The chromagen 4 chloro-1 napthol (0.3%) and 0.001% hydrogen peroxidase in water were added until colour development. Immunoblots were then washed 3 x 10 min with gd water and air dried.

2.10. **Immunoabsorption Affinity Chromatography.**

(i) Column preparation.

Cyanogen bromide (CNBr) activated sepharose 4B beads (Pharmacia, Sweden) were washed and swollen for 15 min in 1M HCl on a sintered glass filter (porosity, G3). The gel was then washed with sodium carbonate coupling buffer pH 8.5 (Appendix) (5ml per gram dry gel). Anti-54 kDa IgG and anti-62 kDa IgG were dissolved in coupling buffer (1 vol:1 vol) and mixed with the gel suspension in an end-over-end mixer, for 2 hours at RT. Excess uncoupled IgG was removed from the gel by four alternate washes with sodium acetate buffer, pH 4 (Appendix). The sepharose gel and IgG solution was transferred to 0.2M glycine pH 2.0 and mixed for 2 hours at RT in order to saturate any

unbound sites on the beads. The glycine blocking solution was then removed by repeated washing the gel with coupling buffer. The protein-sepharose conjugate column was stored at 4°C with coupling buffer, and 0.2% sodium azide.

Immunoaffinity columns were prepared for the isolation of specific infected membrane proteins reactive with IgG from antisera raised against membrane glycoproteins of 54 and 62 kDa. Protein A affinity purified IgG was conjugated to CNBr-activated Sepharose 4B at a concentration of 1 mg anti- 54 kDa IgG/ml beads and 1.43 mg anti- 62 kDa IgG/ml beads. Detergent solubilised plasma membrane proteins (0.4% sodium deoxycholate, Section 2.4.1) from uninfected HEF at a concentration of 0.56 mg of protein/ml for membrane and HCMV infected HEF at a concentration of 0.43 mg of protein/ml were passed through separate columns. The membranes were passed through the columns ten times to ensure maximum binding of all antigen. Unbound protein was removed by passing coupling buffer through the column until the absorbance of the eluate read at zero at an absorbance of 280nm. Plasma membrane proteins in each sample reactive with the specific IgG's were eluted with ethylene glycol: 0.1% Triton X-100 (50:50, v:v), pH 11.5 until the absorbance at 280nm read near zero. The protein eluates were dialysed against five changes of PBS, pH 7.4, and then concentrated to a 100ml in a Minicon concentrator (Amicon, MA, USA). SDS PAGE sample buffer containing 2ME was added 4 vol to 1 vol of protein eluate and the samples analysed by reducing gel electrophoresis (Section 2.5).

2.11. **Radioisotope labelling.**

(i) Metabolic labelling of plasma membranes with L-^{35}[S] methionine.

L-^{35}[S] methionine *in vivo* cell labelling grade (1330 Ci/mmol) (Amersham,UK.) was used to label HCMV infected fibroblasts. In order to establish the time at which HCMV induced glycoproteins appeared in the infected cells and more specifically the plasma

membranes, infected monolayers of fibroblasts were labelled at 12 h., 48 h. and 72 h. post infection for 4 h. Cells were washed with methionine-free MEM , and then pre-incubated with MEM containing 10% of the normal methionine concentration, 2mM glutamine and 2% FCS for 3 hr before adding radioisotope at a concentration of 50μCi/ml (i.e. 500 μCi/10^7 cells). Plasma membrane proteins and whole cell proteins were then isolated, and treated with immunoprecipitation extraction buffer (Section 2.4).

(ii) Labelling of virion envelope proteins with ^{125}I.

Virions and detergent or osmotically extracted virion envelopes (Section 2.3.2) were labelled with ^{125}Iodine using the chloramine-T-method as given by Hunter and Greenwood, (1962) and Strange, *et al.*, (1971). Virion envelopes (100 μl; approximately 25 μg protein)was reacted with 100 μl of sodium phosphate (0.5M, pH 7.5), 5μl of chloramine-T (1mg/ml) and 250 μl ^{125}I (Amersham,U.K.) for 1 min. Sodium metabisulfite (5 μl;3mg/ml) were added to stop the reaction. The volume of labelled proteins was reduced to 50 μl in a in Speed-Vac concentrator (Savant Instruments Inc.). For some samples, 50 μl of SDS PAGE sample buffer was added, and electrophoresed on SDS polyacrylamide gels (Section 2.5). The concentrated labelled proteins were also used directly as a source of antigen in immunoprecipitation experiments (Section 2.12).

I2.12. Immunoprecipitation Procedures.

(i) Immunoprecipitation with *Staphyloccocus aureus*:.

This procedure was based on the method given by Blanton and Tevethia (1981). Plasma membrane extracts 500 μl were incubated with 10-20 μl of IgG from antisera raised against infected membrane glycoproteins of 54, 62 and 94 kDa for 2h at 4^{o}C. A 10% suspension of *Staphylococcus aureus* (100μl) was added to the above suspension and the antigen-antibody complexes adsorbed to the *S.aureus* by incubation at room temperature

(RT) for 20 min with constant end over end mixing. The suspensions were centrifuged at 3,000 rpm for 5 min in an Eppendorf microfuge and the pellets washed four times by resuspension and centrifugation as described above, using 1 ml of washing buffer (0.5M lithium chloride, 0.1M sodium chloride, 0.05M tris-HCl pH 7.4 and 1% Nonidet P40 (v/v)). The pellets were resuspended in 50 µl of electrophoresis sample buffer and incubated at 100°C for 3 min. The bacteria were pelleted by centrifugation at 3000 rpm for 5 min and the supernatants analysed by SDS-PAGE.

(ii) Immunoprecipitation with Protein A Sepharose.

The procedure was based on methods used by Rasmussen *et.al..*, (1984) and Gretch *et.al.*, (1988b). L-^{35}[S] methionine labelled plasma membrane extracts isolated from 175 cm^2 tissue culture flasks by FMA extraction or two phase dextran polyethylene glycol polymer systems (Section 2.3.1) were resuspended in 100 µl of immunoprecipitation extraction buffer containing 1% NP40 and 1% sodium deoxycholate and incubated for 30 min at 4°C with sporadic agitation.

The plasma membranes or cell lysates were clarified by centrifugation at 30,000 rpm in a TLA 100,3 rotor and then incubated with human IgG(Dakopatts, Denmark) (50µg/100µl)) for 4 h at 4°C. Human Fc receptor (43 kDa) and non specific immune complexes were removed by absorption to Protein A-Sepharose beads ; 50 µl of a 50% (v/v) suspension in buffer A (Appendix) for 1 h at 4°C and then centrifuged at 3,000 rpm for 2 min. The resulting supernatants were then incubated with specific antisera (Section 2.6.2) for 12 h at 4°C on a vertical end over end mixer to ensure thorough mixing. Samples were spun at 3,000 rpm for 10 min to remove non specific protein aggregates and the specific immune complexes adsorbed from the supernatents as described above. Protein A sepharose beads were washed three times with PBS pH 7.4, containing 0.1% NP40 and once with gd water. Specific antigens were eluted by the addition of 200 µl 0.1M-glycine-HCl pH 2.3 to each pellet, which was then vortexed for 30 sec and shaken

vigorously for 10 min. Sodium hydroxide (0.1M; 5 µl-10 ml) was added to titrate the eluants to neutral. To concentrate the antigen samples, they were freeze dried and then resuspended in 50 µl of SDS PAGE reducing or non reducing sample buffers for SDS PAGE analysis.

2.13. **Fluorography.**

After reducing and non-reducing SDS PAGE, gels were washed for 10 min in gd water to remove surface SDS, and two different methods of treating the gels with scintillants to enhance the isotope message were used. All immunoprecipitation experiments carried out with *S.aureus* were treated with the fluorographic procedures outlined by Bonner and Laskey (1974). Water was removed from the gels by two washes in $MeSO_4$ of twenty minutes each. The gels were then transferred to a 10% w/v solution of 2.6-diphenyloxazole (PPO) (Merck) in $MeSO_4$ for 2-3 h. The PPO/$MeSO_4$ solution was decanted and the excess $MeSO_4$ removed by washing in running water for 3h. Gels were dried onto filter paper in a Pharmacia slab dryer and placed in contact with Fuji RX X-ray film at -70^{o}C.

Immunoprecipitation experiments performed with protein A Sepharose as the immunosorbent were imbibed with Amplify solution (Amersham) for 30 min on a rotary shaker before transferring the gel to a Pharmacia or Bio-Rad slab dryer. Dried gels were placed in contact with Fuji-RX-X-ray film or Amersham Hyperfilm MP, with or without calcium tungstate X-ray intensifying screens (Kodak-X-omatic; Agfa-Gevaert) (Laskey, 1980) at -70^{o}C. Radioactively exposed film was developed with Kodak D19 high contrast developer and the absorbance profile of the film image recorded by laser densitometry (LKB, 2222-020 Ultrascan XL, Bromma, Sweden). The molecular weights of the labelled antigens were calculated using a mixture of ^{14}C-labelled proteins. Two sets of calibrated markers were used, ^{14}[C]labelled protein molecular weight markers (Amersham), and

^{14}C-labelled Rainbow^{TM} Protein Molecular Weight Markers (Amersham) (Appendix).

2.14. Photographic Procedures.

2.14.1. Image Subtraction Analysis.

Mask films of autoradiographs of 35 [S] methionine labelled plasma membranes and cell lysates from HCMV infected and uninfected cells were made by using subtraction film (Cronex, Du Pont). The mask film is a copy of the base film which has the densities reversed. When placed over the base film none of the images can be seen. Masks of protein profiles of plasma membranes and cell lysates from uninfected cells were registered over protein profiles of plasma membranes and cell lysates from infected cells and prints made of the addtional bands present. All bands present in the final print represent HCMV induced proteins.

2.14.2. Image Enhancement Analysis.

Autoradiograghs were processed into a video display unit with a Pansonic WV CD50 camera and the information digitized using either Image Action or Java image analysis programmes (Jandel Scientifics, USA.).

RESULTS

3.1. Plasma Membrane Proteins of HCMV-Infected and Uninfected HEF

Two methods of membrane isolation were employed for the further characterization of plasma membrane glycoproteins induced by HCMV infection in fibroblasts. The FMA extraction procedure (Section 2.3.1.) was one method used for the isolation of plasma membranes from infected and uninfected fibroblasts grown as adherent monolayer cultures. After the removal of the upper plasma membranes from the cells, the cell organelles remained associated with the cell (Figure 1).

Approximately 80-100 μg of plasma membrane protein was obtained from a 175 cm^2 monolayer of HEF. Solubilized membranes obtained from uninfected and infected cells (approximately 100μg protein) prepared as in Section 2.4.2. were electrophoresed under reducing conditions to ascertain the novel HCMV-induced plasma membrane glycoproteins. At 72 h after infection proteins of M.W. 30, 54, 62, 72, and 94 kDa were identified on 10% reducing SDS gels visualised with Coomassie blue R250 (Figure 2). These proteins were consistently identified from ten different FMA plasma membrane isolations when analysed by reducing SDS PAGE under reducing conditions. The molecular weight ranges are in agreement with those of plasma membrane glycoproteins isolated from HCMV infected fibroblasts by Sullivan-Tailyour and Garnett, 1986, who used this membrane preparation procedure.

The two-phase polymer purification procedure for plasma membranes, (Section 2.3.1.) was used as an alternative membrane isolation procedure to the FMA technique. This method allows for the separation of cellular membranes on the basis of differences in their surface properties.

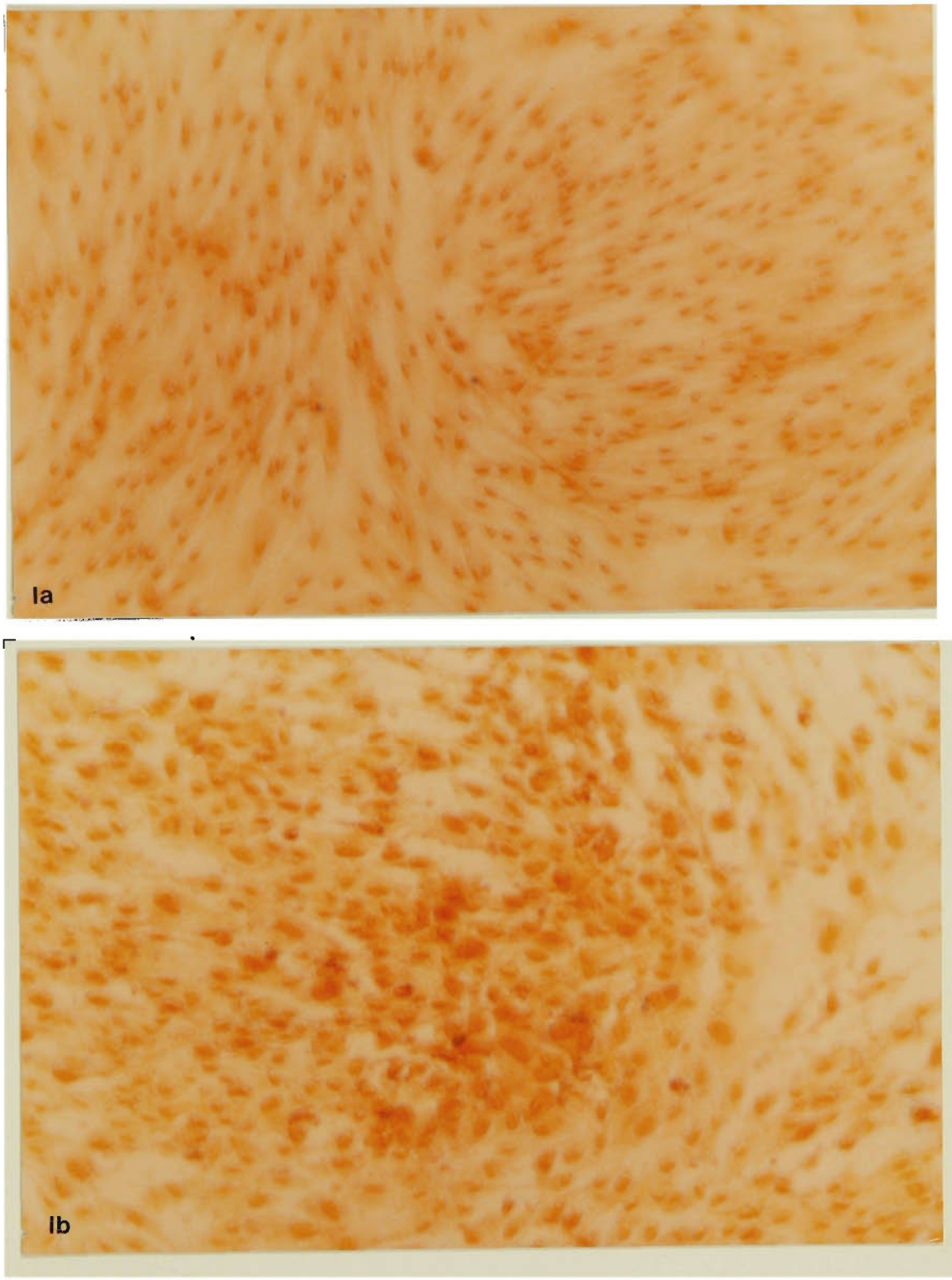


Figure 1. Light micrographs of uninfected and HCMV infected HEF, following plasma membrane removal. The upper membrane surface was stripped from the adherent monolayer of cells using the FMA extraction procedure. The cytoplasmic organelles are left intact.

(1a) Uninfected HEF (x 50).

(1b) HCMV infected HEF at 72 h after infection (x 50).

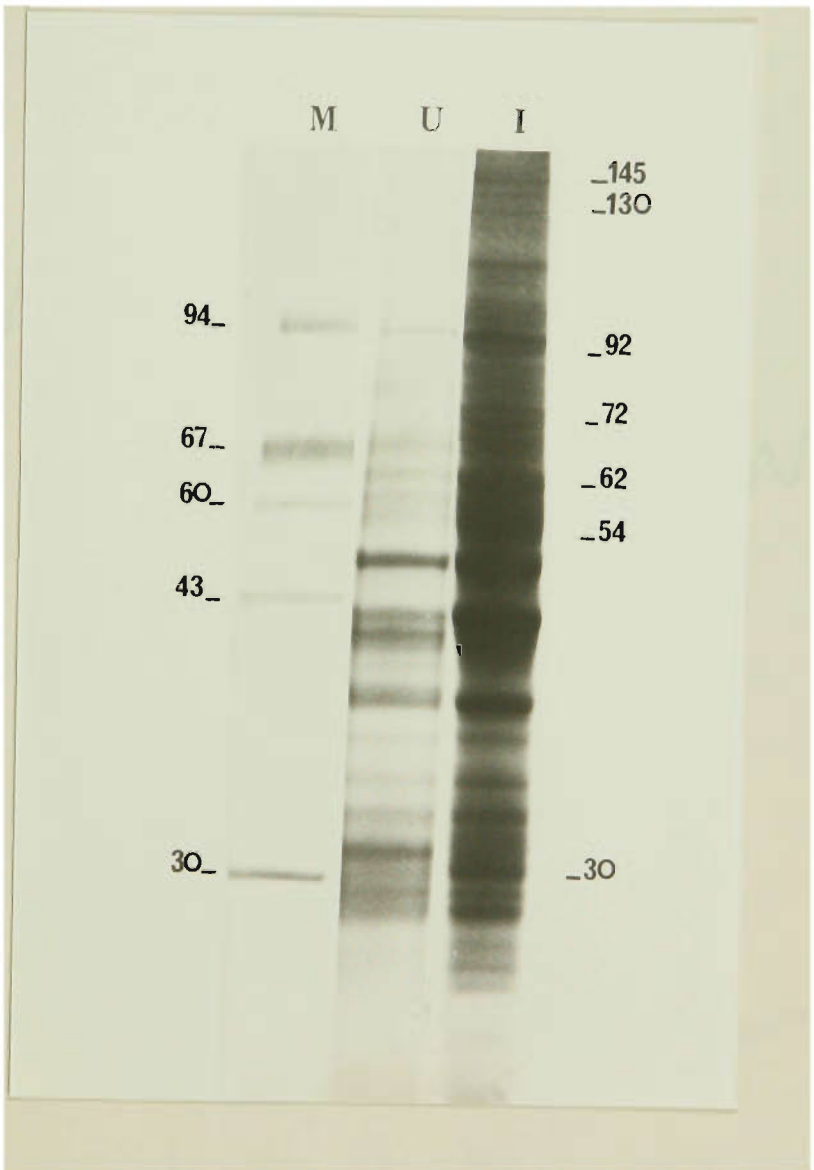


Figure 2. Photograph and densitometer scan of 10% reducing SDS PAGE analysis of plasma membranes isolated from infected (I) and uninfected (U) human embryonic lung fibroblasts (HEF) 72 hours after infection by FMA extraction. Molecular weights ($X10^3$) of markers (M) and proteins induced by virus infection are indicated.

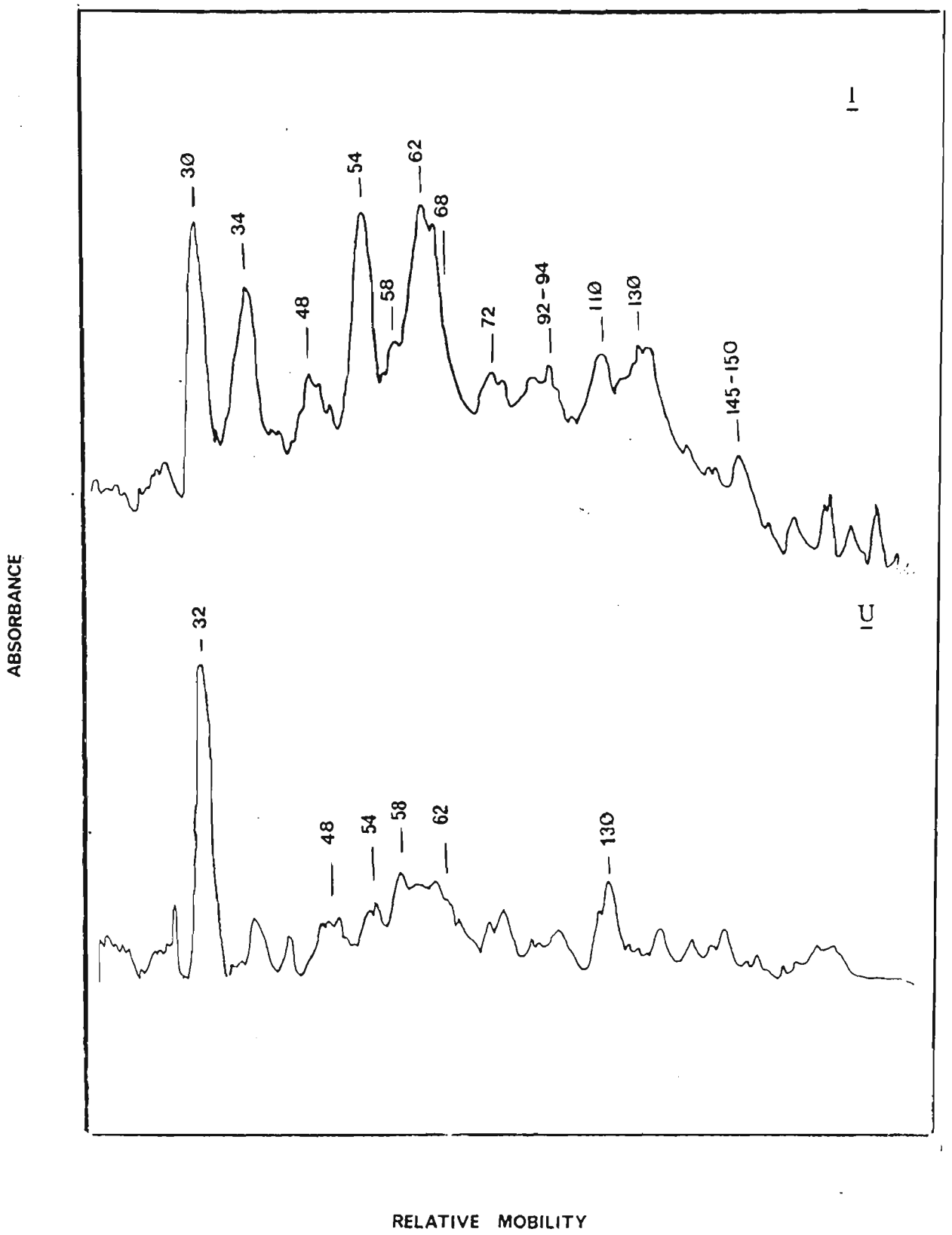


Figure 2 continued.

The protein yield of membraneous material obtained by two-phase polymer extraction was in the range of 50 μg of protein from a 175 cm^2 monolayer of cells. This was consistently lower than that obtained for the FMA extraction procedure.

Plasma membranes isolated by FMA extraction have been previously assesssed for purity by transmission and phase contrast microscopy. It was shown that this method yielded an undamaged membrane fraction with neglible cytoplasmic contamination (Mc Clure *et al* ., 1979; Sullivan Tailyour, 1986). To check that the two phase polymer extraction procedure was producing plasma membrane fractions, Na^+, K^+-ATPase activity was assessed, this being a marker for plasma membranes. The procedure cannot be used with the FMA preparations, as the FMA inactivates enzymes. The enzyme activity of the plasma membrane fraction was compared to that of the whole cell homogenate. Table V shows the results of three experiments in which specific activities of this enzyme were measured in the homogenate and plasma membrane fractions. The average specific activity of Na^+, K^+-ATPase in the membrane fraction was 2.43 μ moles phosphorous (P) released/mg protein/hr and is in the range of Na^+-K^+-ATPase activity reported for enriched plasma membrane fractions (Warren *et al* ., 1967 and Brunett and Till, 1971).

Experiments were undertaken to ensure that the protein profiles of membranes from infected cells were similar using the two extraction procedures. Metabolically labelled plasma membranes were purified by both the two-phase polymer and FMA extraction procedures and fractionated on 10%-20% gradient reducing SDS PAGE (Figures 3 and 4, respectively). The results given here are taken from 2 and 3 different isolations for the FMA and two phase polymer procedure respectively. Both methods of plasma membrane isolation yielded protein profiles in which HCMV-induced membrane proteins of M.W. 30, 54-55, 62, 72, 92, 130 and 145 -150 kDa could be consistently identified.

After the removal of the non-adherent plasma membranes by the FMA procedure, L-^{35}[S] methionine-labelled whole cell constituents that remained attached to the flask, including

Table V.

Specific Activities of Na^+, K^+-ATPase in Plasma Membranes and Whole Cell Homogenates.

Expt. No.	Homogenate	Plasma Membrane
1	0.41	1.2
2	0.30	1.4
3	0.69	4.7

Specific activity of Na^+, K^+-ATPase in plasma membranes isolated from fibroblasts by two phase polymer purification and enrichment procedures and whole cell homogenates is expressed as μ moles (P) released/mg protein/hr.

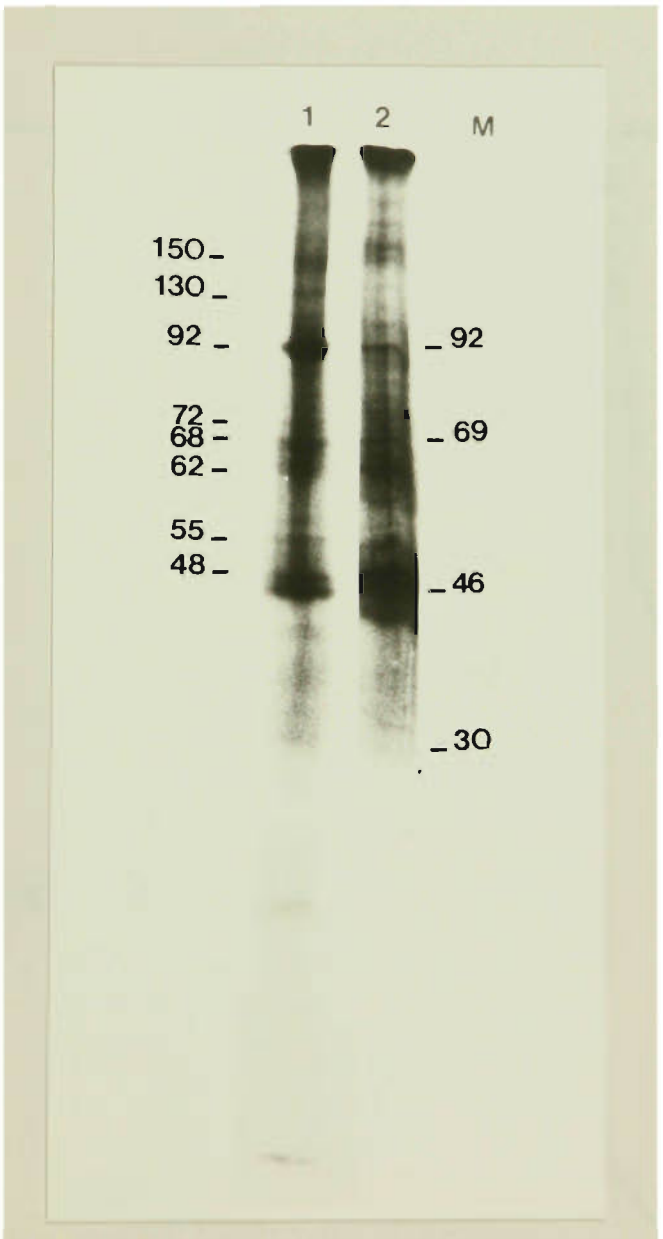


Figure 3. Autoradiograph and densitometer scan of 10%-20% reducing SDS PAGE analysis of L^{35} [S] methionine labelled plasma membrane proteins from HCMV infected HEF (lane 1) and uninfected HEF (lane 2), extracted 72 h after infection and purified by two phase polymer centrifugation. Apparent molecular weights ($X10^3$) of proteins induced by virus infection and molecular weight markers (M) are indicated.

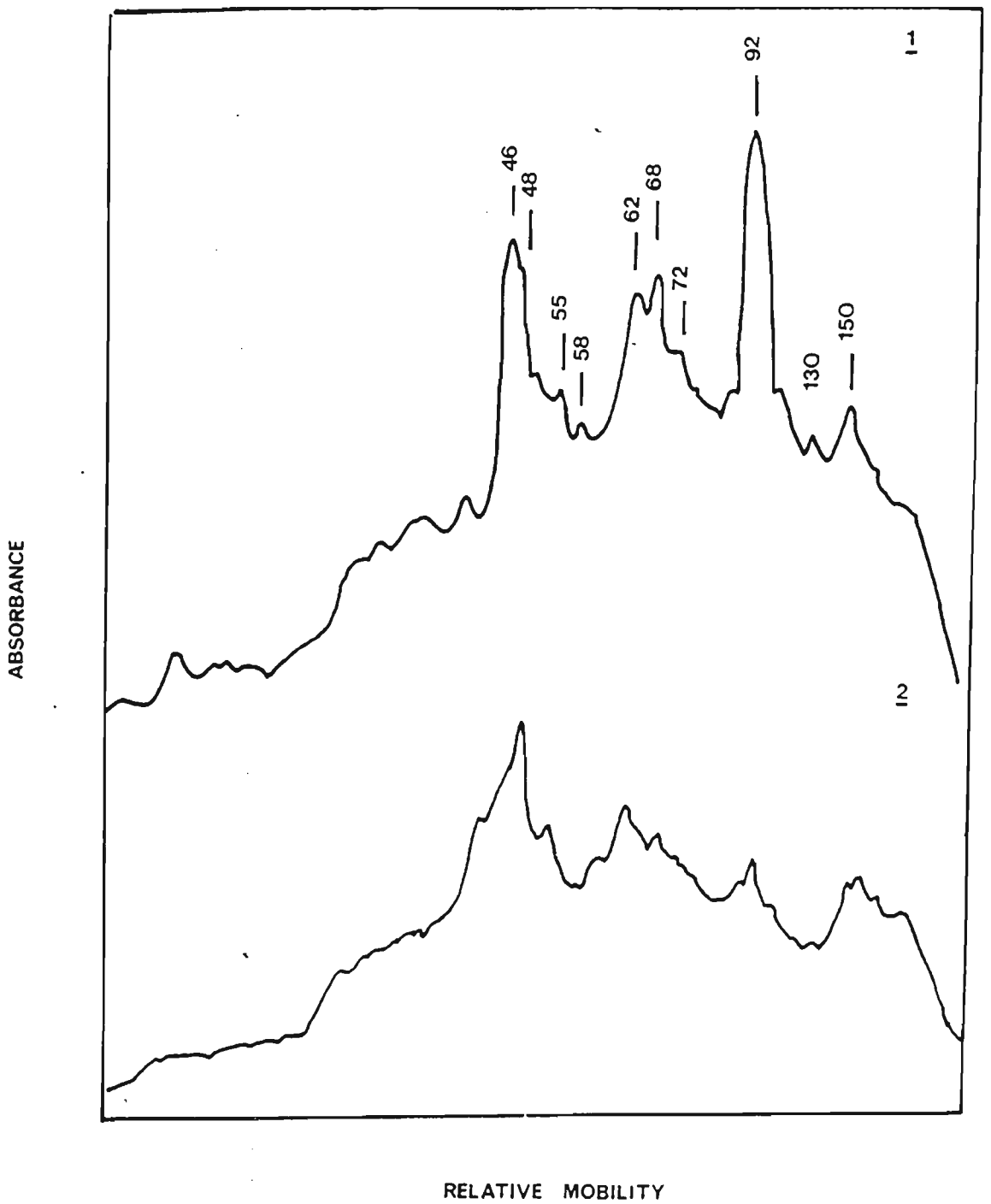


Figure 3 continued.

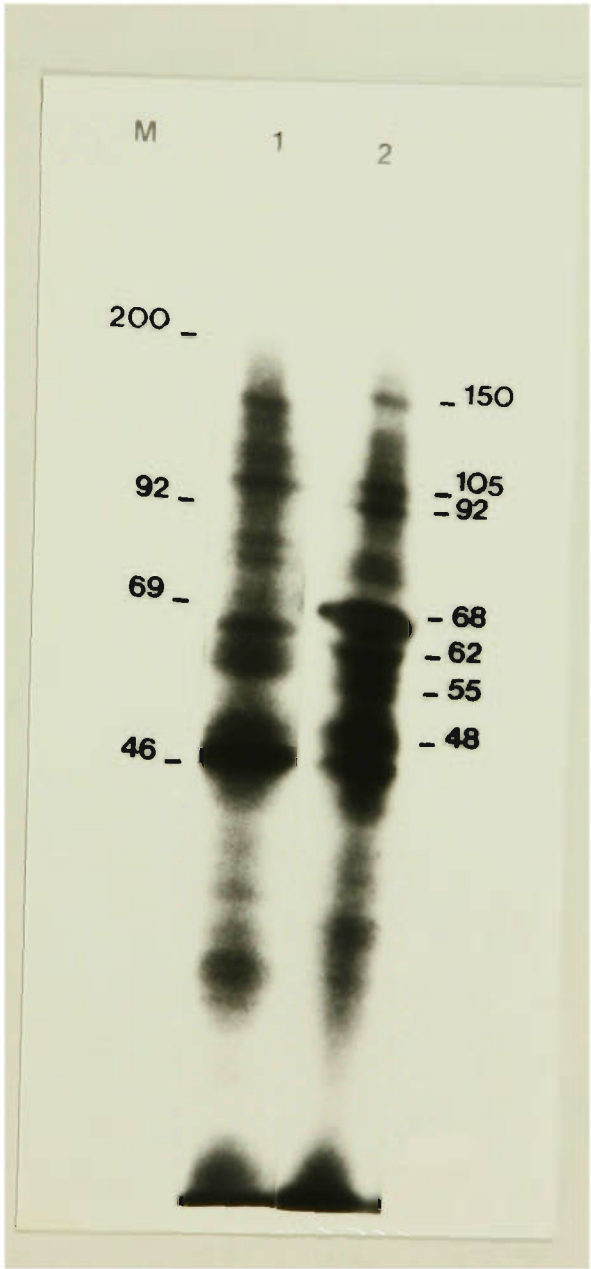


Figure 4. Autoradiograph and densitometer scan of 10%-20% reducing SDS PAGE analysis of L^{35} [S] methionine labelled plasma membrane proteins of HCMV infected cells (lane 2) and uninfected cells (lane 1) isolated by FMA extraction. Molecular weights ($X10^3$) of viral induced proteins and markers (M) are given.

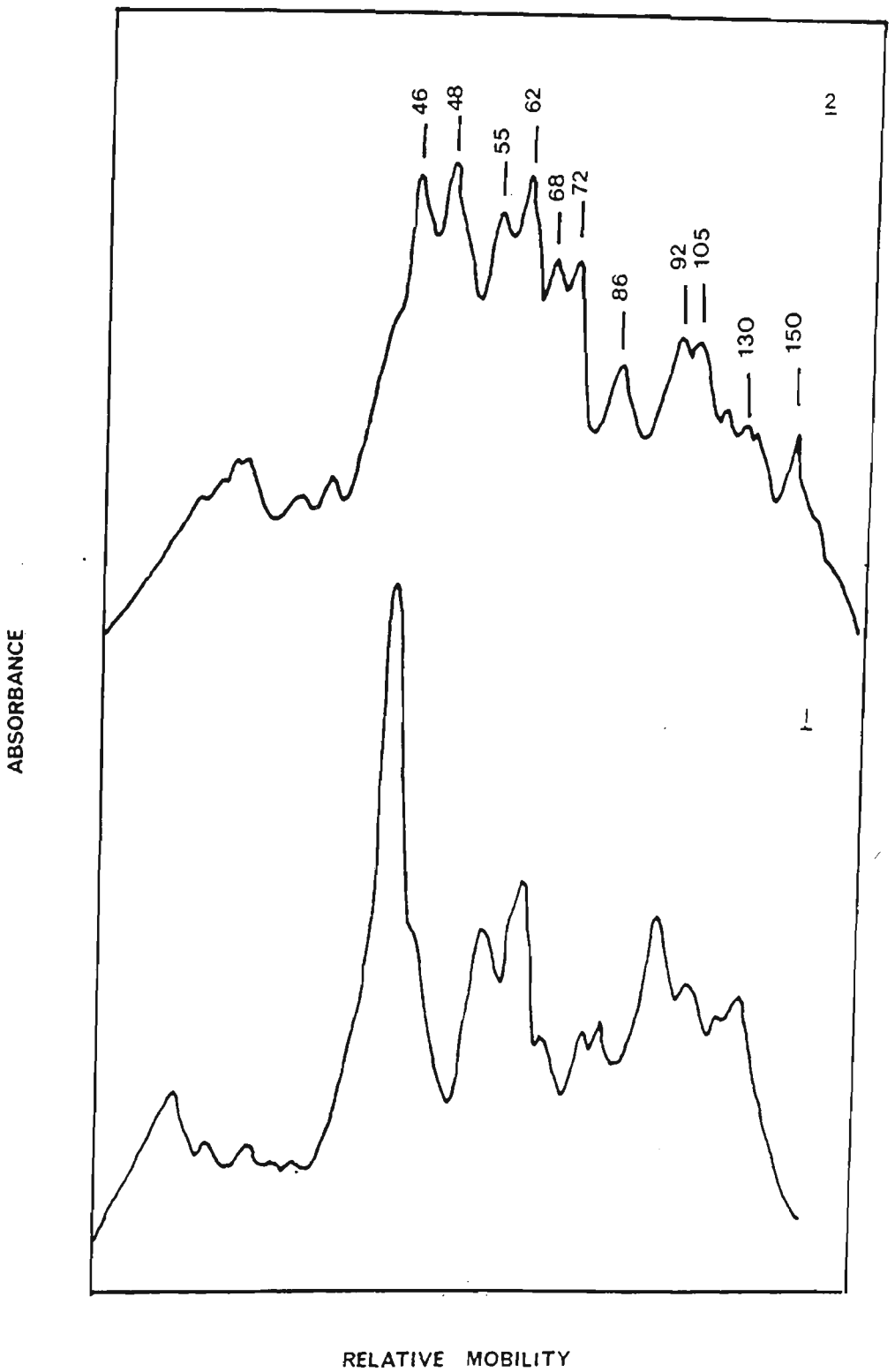


Figure 4 continued.

the underlying plasma membranes, were solubilised with 1% NP 40 and 1% sodium deoxycholate (Section 2.4.2). These proteins were fractionated on 10%-20% gradient reducing SDS gels (Figure 5). The labelled-protein fraction contained a relatively high concentration of HCMV-induced proteins of M.W. 55, 68, 72, 92 and 150 kDa.

Autoradiograph image subtraction analysis (Section 2.14.1) of fractionated protein profiles of uninfected and infected plasma membranes isolated by the FMA procedure (Figure 4) and uninfected and infected whole cell lysates prepared according to procedures given in Section 2.4.2. (Figure 5) was carried out. The result of the subtraction of HCMV-uninfected cell plasma membrane protein profiles from the fractionated protein profile of plasma membranes from infected cells indicates that HCMV-induced proteins of M.W. 26, 38, 48, 55, 62, 68, 92, 105, 130 and 150 kDa exist in the plasma membranes of infected cells (Figure 6, lane 1). When similar procedures were used to analyse the infected and uninfected cell lysates, the virus-induced proteins found in abundance in the infected cell after removal of the plasma membranes, by FMA extraction,were of M.W. 24 , 46 , 48, 68 , 92 and 130 kDa (Figure 6, lane 2).

3.2. Antisera Specific for HCMV-Induced Plasma Membrane Proteins

Several samples of plasma membrane proteins isolated from infected cells by FMA extraction were electrophoretically separated on 10% reducing SDS gels and stained with water soluble 0.1% CBB G-250. Proteins bands of M.W. of 54, 62 and 94 kDa were excised directly from the gels, and used to immunise New Zealand white rabbits (Section 2.5.1). The antisera were used in a number of studies in order to further characterise the specific proteins to which the antisera were raised.

3.2.1. Immunobinding Assays

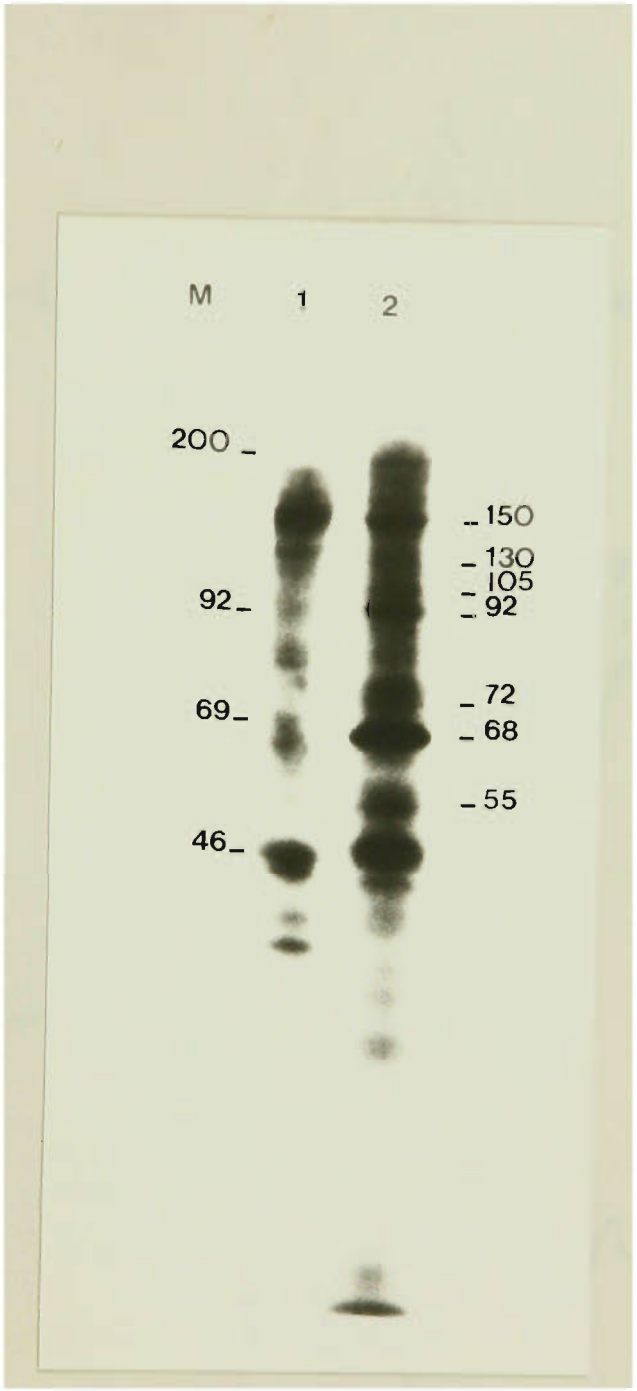


Figure 5. Autoradiograph and densitometer scan of 10%-20% reducing SDS PAGE analysis of L^{35} [S] methionine labelled whole cell proteins of HCMV infected cells (lane 2) and control cells (lane 1). After isolation of the upper surface plasma membranes by FMA extraction, the remaining cell constituents including the underlying plasma membranes were scraped off into solubilising buffer containing 1% NP 40 and 1% sodium deoxycholate, incubated at 4 C for 30 mins and centrifuged at 100,000g for 30 mins before application to the gels.

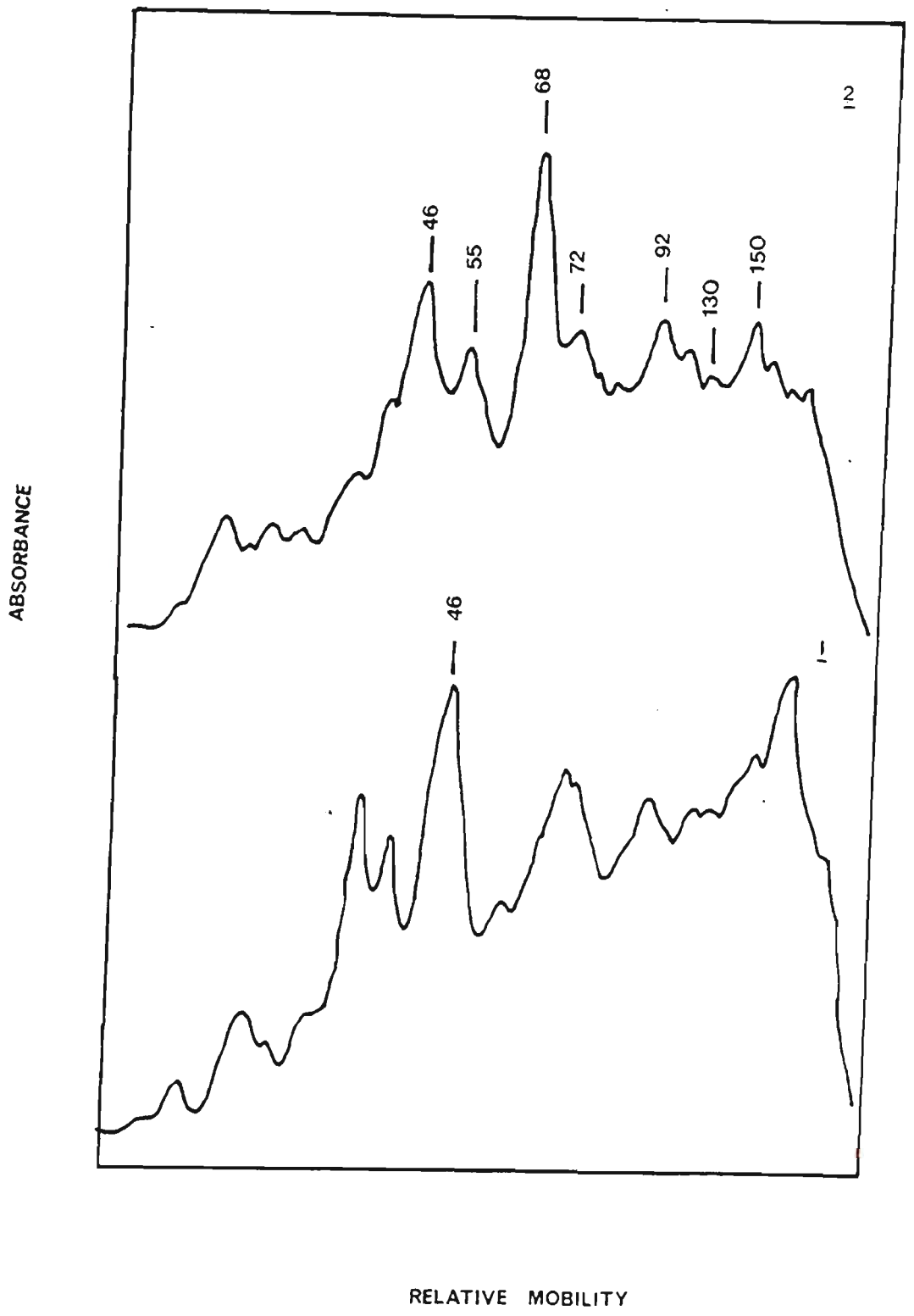


Figure 5 continued.

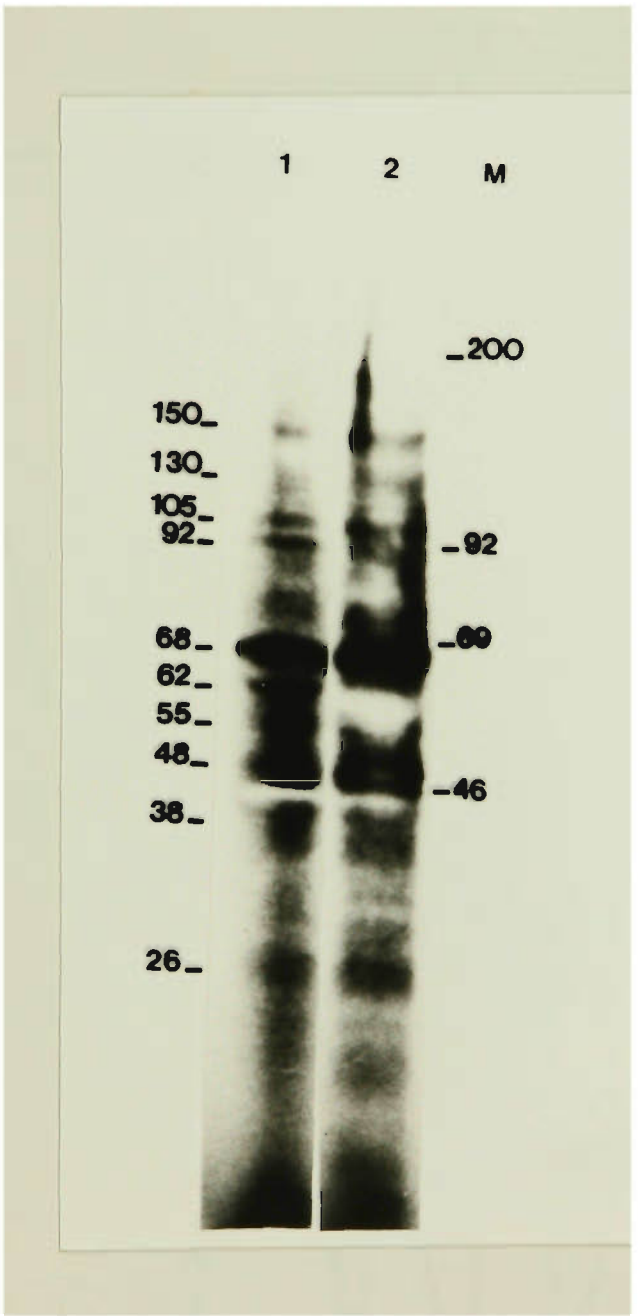


Figure 6. Autoradiographs and densitometer scans of subtraction analyses of 10%-20% reducing SDS PAGE of L^{35} [S] methionine labelled plasma membranes and whole cell protein of HCMV infected and uninfected cells. Lane 1 represents the subtraction of uninfected cell plasma membrane proteins (from Fig. 4, lane 1) from HCMV infected cell plasma membrane proteins (from Fig. 4, lane 2), and lane 2 that of uninfected cell proteins (from Fig. 5, lane 1) from HCMV infected cell proteins(from Fig. 5, lane 2)

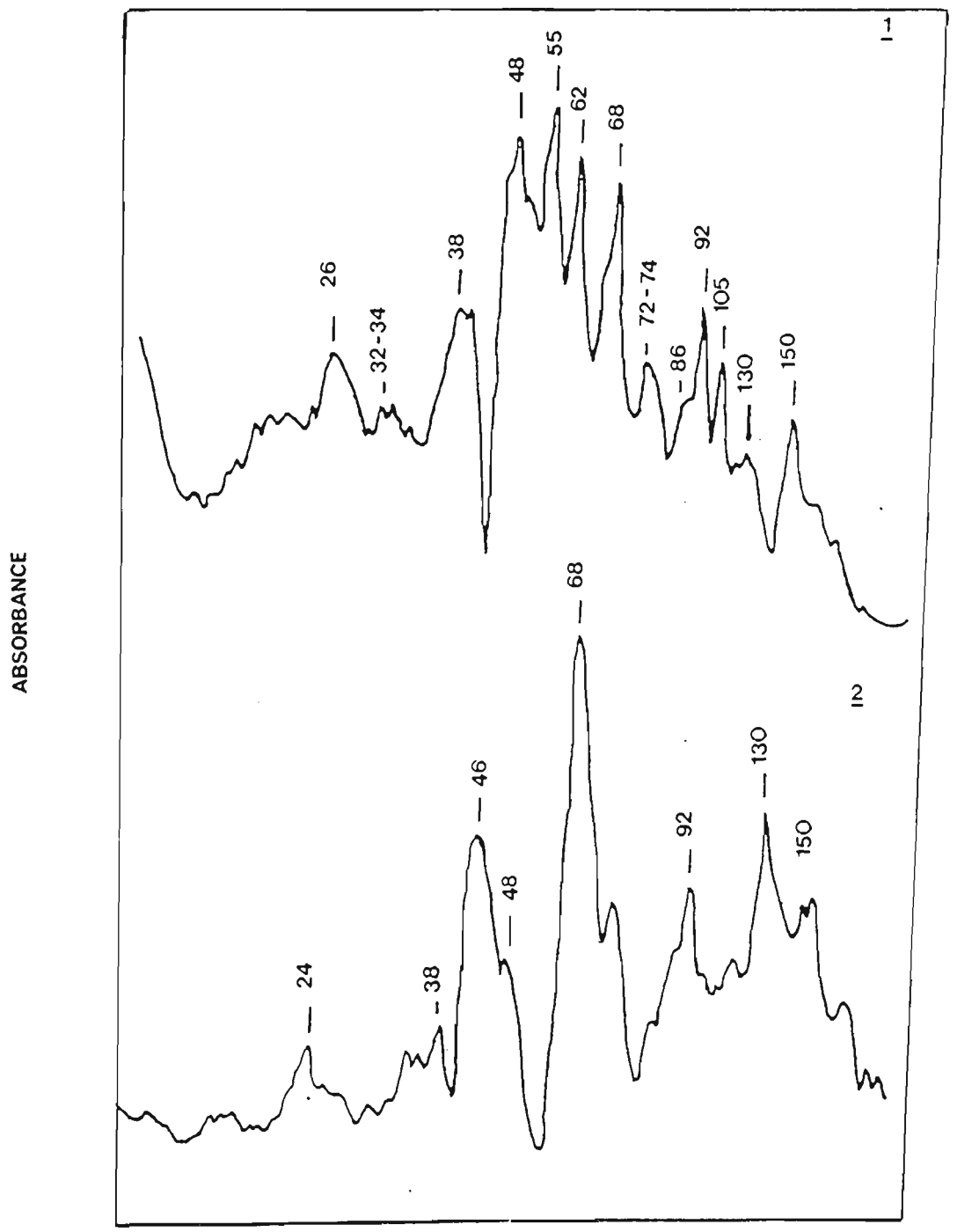


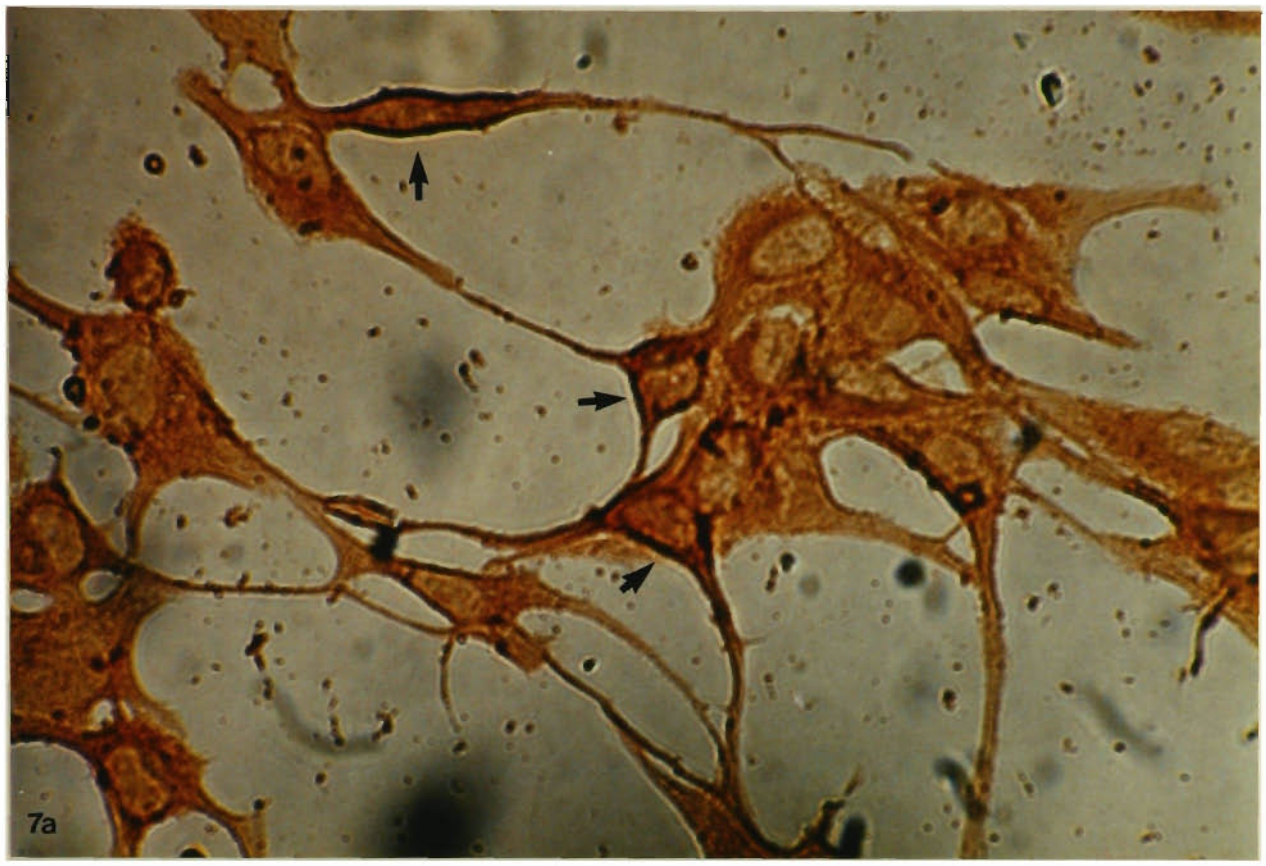
Figure 6 continued.

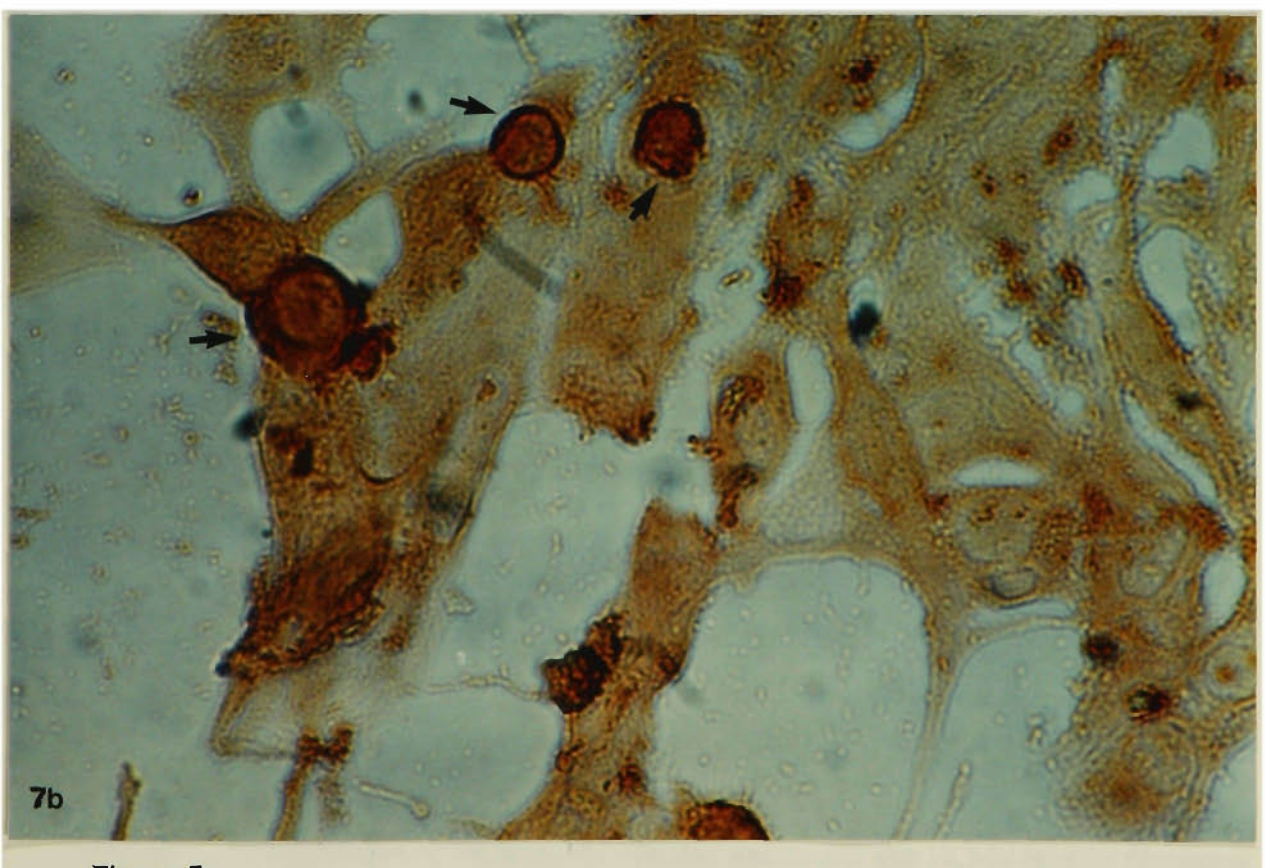
The sites to which antisera, raised in rabbits, against plasma membrane proteins of M.W. 54, 62 and 94 kDa, bound in HCMV-infected cells were localised using peroxidase conjugated goat anti-rabbit IgG activated with diaminobenzidine (DAB) and hydrogen peroxide. HCMV-infected and uninfected cells were treated with neat, 1:10 and 1:100 dilutions of antisera raised against plasma membrane proteins 54, 62 and 94 kDa to establish the minimum concentration of antisera which gave detectable staining. One in a hundred dilutions for all three antisera gave detectable staining in infected cells at 72h after infection. Neat, 1:10 and 1:100 dilutions of pre-immune rabbit antisera were used as controls to test whether the rabbit antisera cross reacted with uninfected and HCMV-infected fibroblasts and at the 1:100 dilution these were minimally reactive with uninfected and infected fibroblasts (Figure 10). Antisera raised against the 54, 62 and 94 kDa proteins bound to the surface and nuclear membranes of infected cells, and not to those of uninfected cells (Figure 11), indicating the specificity of the antisera for infected cell proteins located on the plasma and nuclear membranes (Figures 7, 8 and 9).

Inverse image enhancement analysis was carried out for Figure 7a (Figure 12a) and Figure 8a (Figure 12b) and inverse photographic analysis on Figure 9b (Figure 13a) and Figure 11 (Figure 13b). Staining of the plasma membranes in Figures 12a and 12b clearly demonstrates the affinity of anti- 54 kDa, and anti- 62 kDa sera for proteins located in the plasma membrane. Intense staining of the cytoplasm and cytoplasmic inclusion bodies in Figure 13a indicates the affinity of the 94 kDa antisera to these sites within the infected cell.

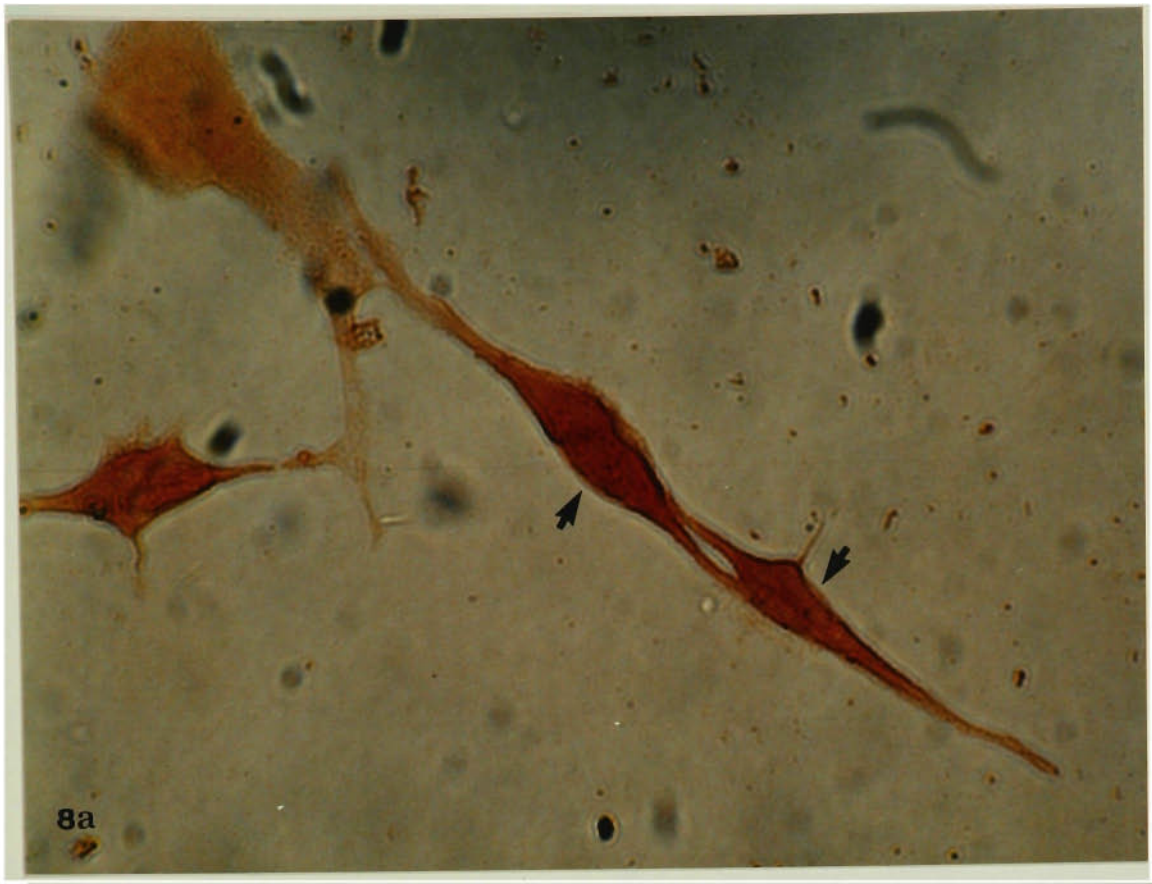
3.2.2. **Neutralisation**

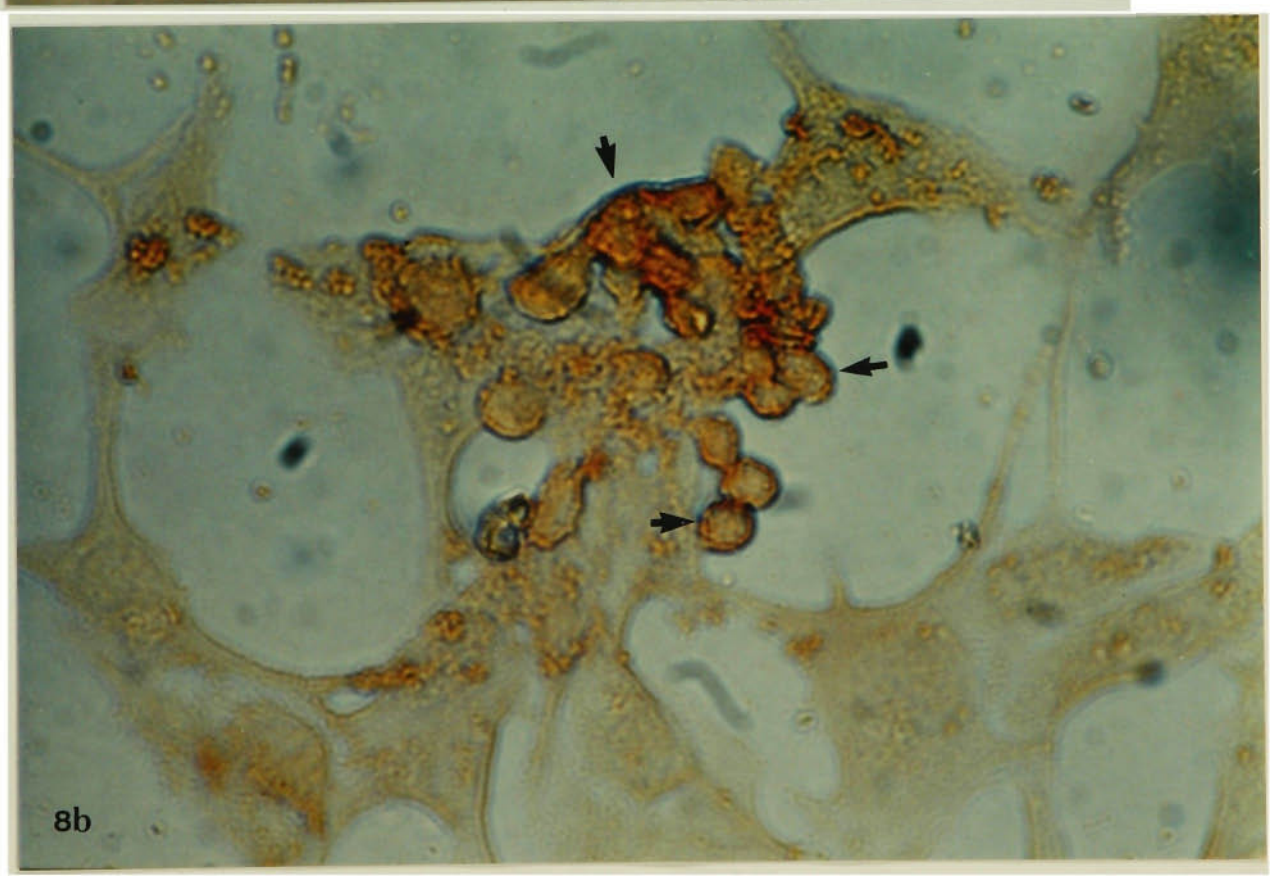
IgG specific for plasma membrane glycoproteins 54 and 62 kDa induced virus neutralisation (Section 2.8.). All neutralization assays were carried out in triplicate. Virus was used at a m.o.i. of 0.2, 2, $2x10^1$, $2x10^2$, $2x10^3$, $2x10^4$, $2x10^5$, $2x10^6$, $2x10^7$ and $2x10^8$ PFU/ml. IgG from antisera raised against the 54 kDa protein and the



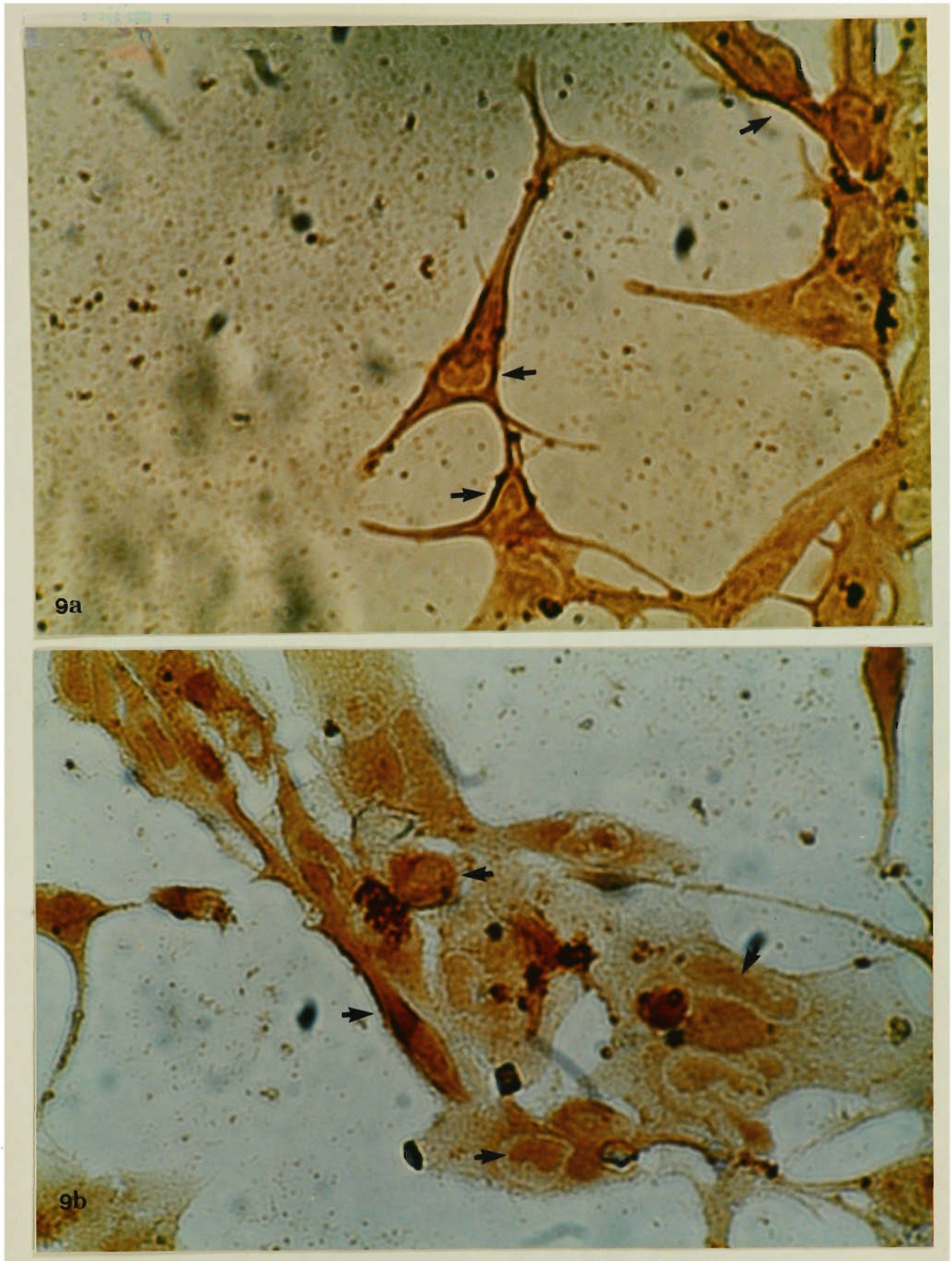


Figures 7a and 7b. 1:100 dilution of rabbit anti-54 kDa sera bound to the surface (a) and nuclear membranes (b) of infected cells. The second conjugate, peroxidase labelled goat anti-rabbit IgG was reacted with diaminobenzidine and hydrogen peroxidase. Brown deposits indicate the sites to which the anti-sera bound (arrows).





Figures 8a and 8b. 1:100 dilution of rabbit anti-62 kDa sera was reacted with HCMV infected fibroblasts 72 hrs post infection. Diaminobenzidine and hydrogen peroxidase was added to the second conjugate, peroxidase labelled goat anti-rabbit IgG. The brown deposits indicate the sites to which the anti-sera bound (arrows). The 62 kDa protein is located in plasma membranes (a) and nuclear membranes (b) of infected cells.



Figures 9a and 9b. 1:100 dilution of rabbit anti-94 kDa sera was reacted with HCMV infected fibroblasts 72 hrs post infection. The second conjugate, peroxidase labelled goat anti-rabbit IgG was reacted with diaminobenzidine and hydrogen peroxide, resulting the deposition of brown deposits where anti-sera bound to the 94 kDa antigen. Strong reactivity with the plasma membranes is indicated in 9a. The cytoplasm and nucleus also stained, 9b.

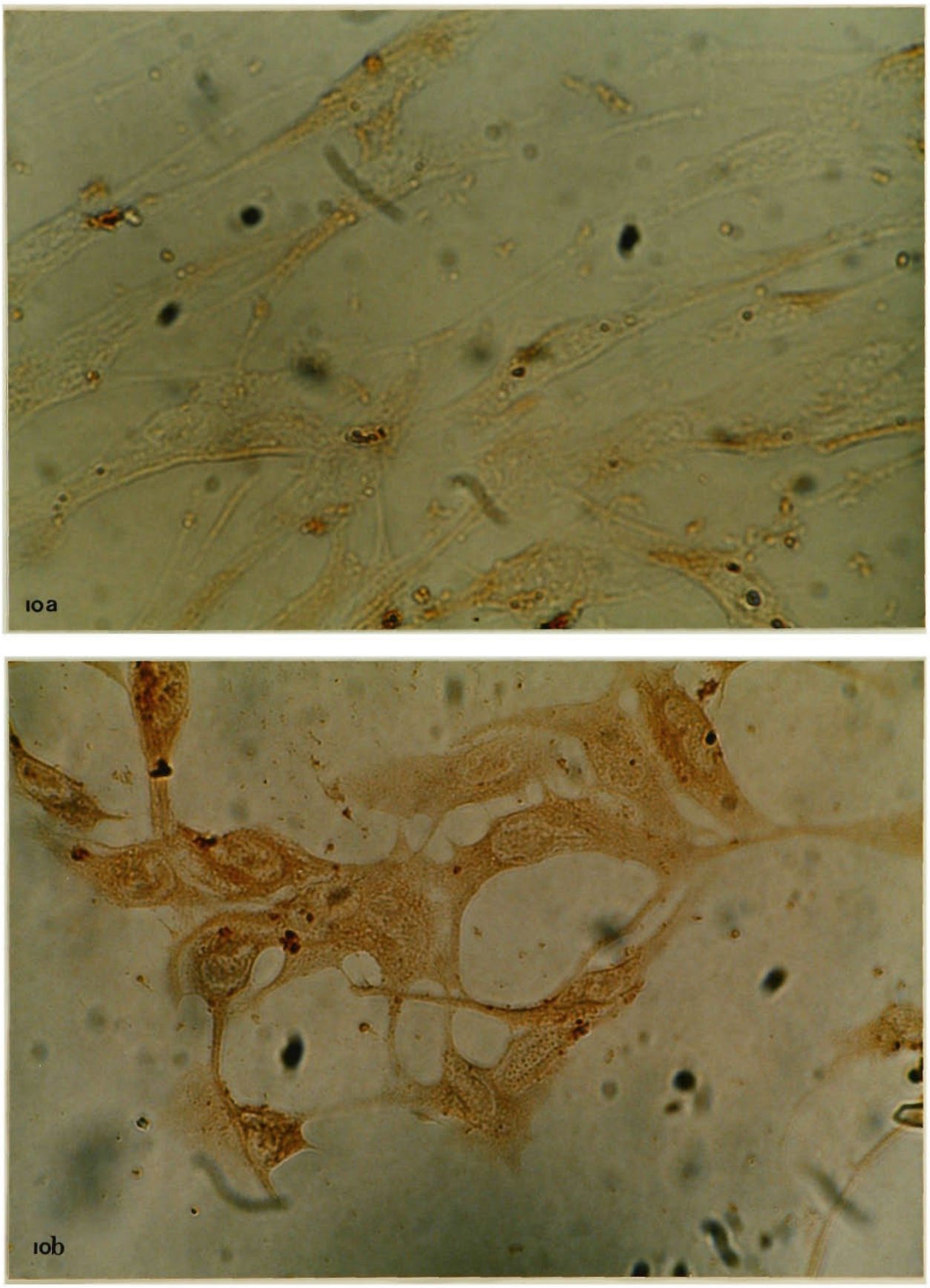


Figure 10a and 10b. 1:100 dilution of pre-immune rabbit anti-sera was reacted with HCMV infected (b) and uninfected fibroblasts (a). Minimal binding of anti-sera was evident.

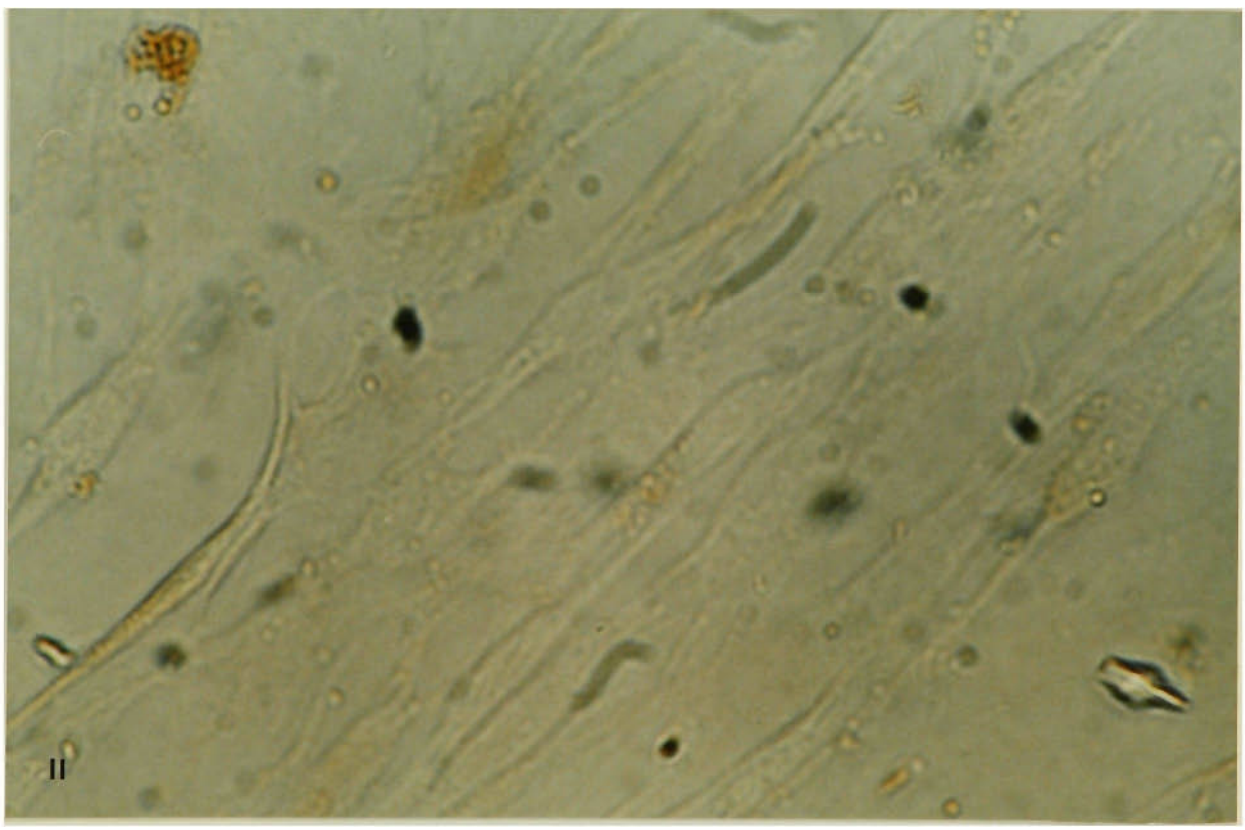
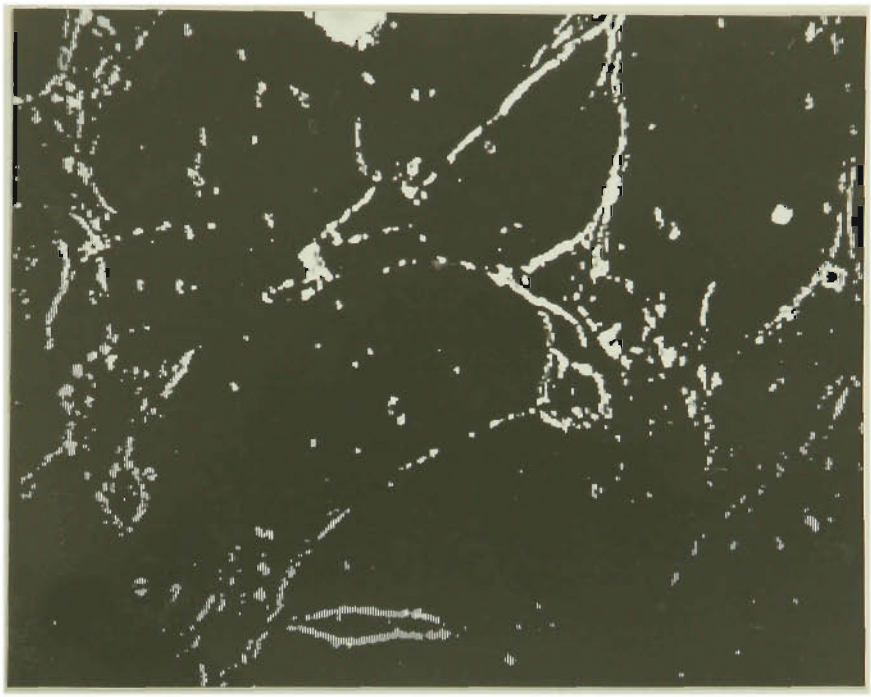
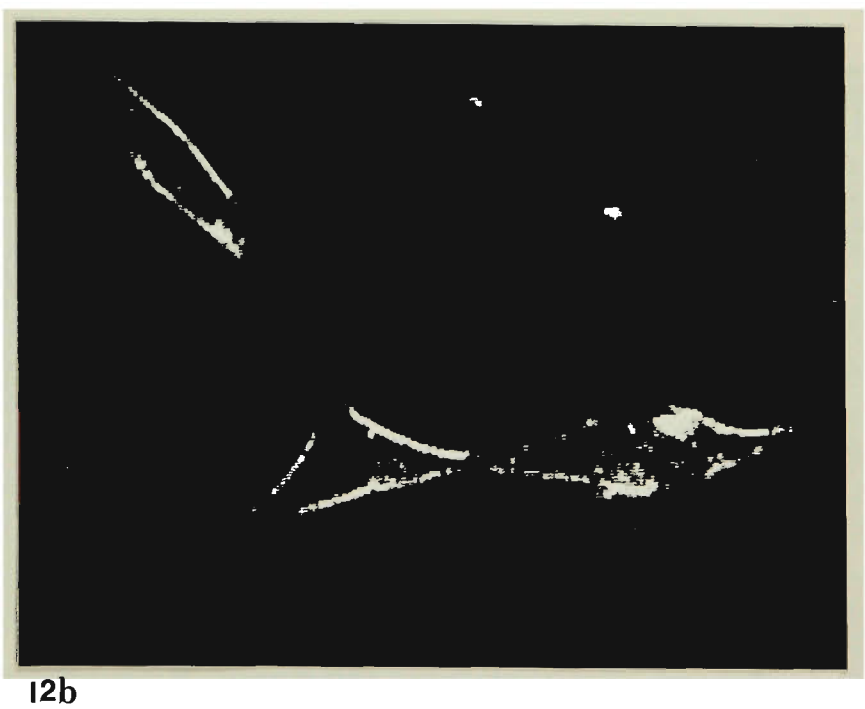


Figure 11. 1:100 dilutions of anti-54 kDa , anti-62 kDa and anti-94 kDa sera were added to uninfected fibroblasts. Only minimal binding of anti-sera was evident, compared to infected fibroblasts reacted with the same antisera.



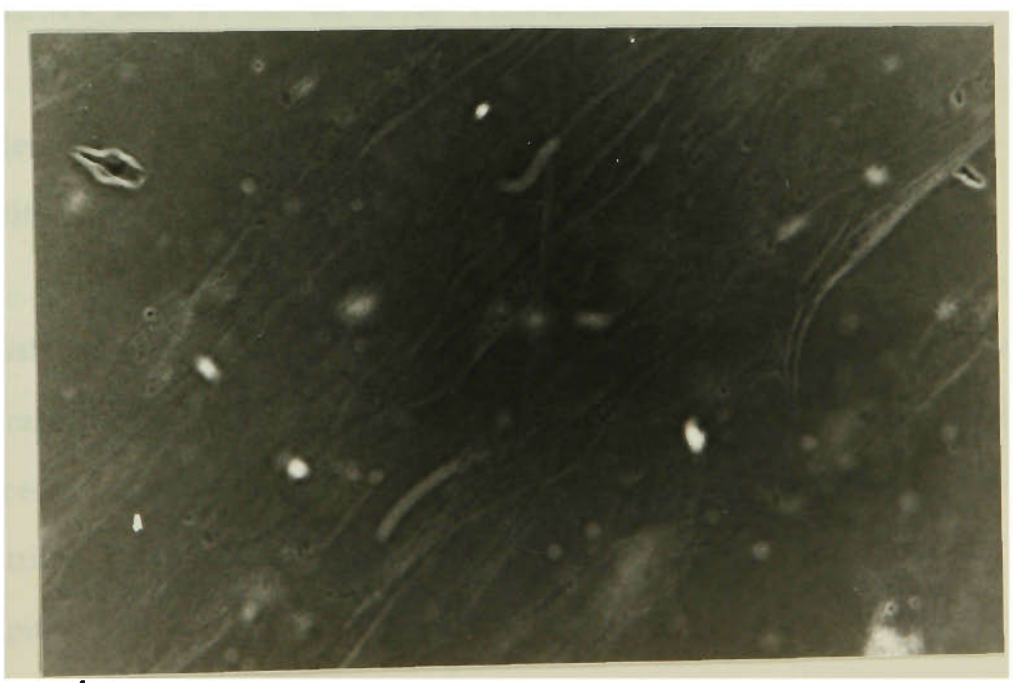
12a



12b

Figures 12a and 12b. Inverse image enhancement analysis of Fig. 7a (infected cell reacted with 1:100 dilution of anti-54 kDa sera ; 12a) and Fig.8a (infected cell reacted with 1:100 dilution of anti-62 kDa sera ; Fig 12b). Binding of anti-sera to the plasma membranes of infected cells can be seen by the outlined image of the cells.

13a



13b

Figures 13a and 13b. Inverse photographic analysis of Fig. 9b (13a) and Fig. 11 (13b). Binding of anti-sera to cells is visualised by white regions, whereas black or grey regions indicate minimal or no binding. In the control, reactivity with anti-54 kDa, anti-62 kDa and anti-94K sera, no binding can be seen, and the outline of the cells is just detectable.

62 kDa protein were used at final concentrations of 20µg and 25 µg respectively, and complement at a final concentration of 2%. IgG from both antisera was able to neutralize over 50% of the virus for m.o.i. of $2x10^5$ through to 0.2 PFU/ml. The addition of complement was also shown to enhance neutralization. A summary of the results is given in Table VI. The percentage neutralization of both these antisera at m.o.i. of $2x10^5$ and $2x10^6$ was approximately 50% and 40% and at higher concentrations of virus it was difficult assess the percentage reduction in the number of CPE in infected cells that were treated with antisera. These results suggest that proteins in the membrane of infected cells are similar to or have antigenic epitopes in common with proteins in the virion envelope. An examination of HCMV virion and virion envelope proteins which react with these antisera was undertaken.

3.3. HCMV and Virion Envelope Proteins.

3.3.1. Reactivity of HCMV Induced Plasma Membrane Proteins with Antisera Raised Against Virions

It has previously been suggested that HCMV-induced plasma membrane proteins react with antisera raised against HCMV virions (Sullivan-Tailyour, 1986). To verify whether this was indeed the case, enveloped virions isolated by 20%-70% linear D-sorbitol gradient centrifugation were used to immunise rabbits, and the antisera used for immunoprecipitation analysis with purified plasma membranes (Sections 2.2.3, 2.6.1 and 2.12).

Table VI.

Percentage Neutralisation of Virus with Anti-54 kDa IgG, and Anti-62 kDa IgG

m.o.i.:	$2x10^4$	$2x10^3$	$2x10^2$
	% Reduction of CPE		
Anti-54 kDa-IgG	83	84	84
Anti-54 kDaIg + 2%C	90	97	91
Anti-62 kDaIgG	82	75	77
Anti-62 kDaIgG+2%C	96	80	94

The neutralization of HCMV virus is expressed as the % reduction of the number of CPE in fibroblasts treated with virus at differnet m.o.i. and anti-54 kDa IgG or anti-62 kDa IgG, in the presence and absence of 2% complement (C).

The anti-54kDa immunoglobulin was at a final concentration of 20µg/ml and the anti-62kDa immunoglobulin at 25µg/ml after addition to the relevant dilution of virus. This equated to a dilution of 1:1000 of the original serum. Preimmune serum at concentrations of 1:10 and 1:100 did not demonstrate any neutralisation.

L-^{35}S methionine-labelled plasma membranes isolated by FMA extraction from uninfected and infected fibroblasts 72h after infection, were reacted with rabbit antisera raised against enveloped virions. The immune complexes were adsorbed with *S. aureus* protein A, and the immunoprecipitates separated on 10% reducing SDS gels and analysed by fluorography (Figure 14). Plasma membrane proteins from infected fibroblasts of M.W. 52-54, 48, 42, 38, 32, 24 and 20 kDa reacted with the virion antisera. Two minor protein bands at 94-96 and 82 kDa were also detected. Proteins of M.W. 52-54, 32 and 24 kDa showed a high affinity for the HCMV antisera. In order to establish whether some of these HCMV induced plasma membrane proteins which are recognised by antisera raised against whole virions were antigenically and structurally similar to virion proteins, proteins from virions, dense bodies and virion envelopes were characterized by SDS PAGE under reducing conditions and then immunoprecipitated with rabbit antisera raised against plasma membrane proteins of 54, 62 and 94 kDa..

3.3.2. **Virions, Virion Envelopes and Dense Bodies.**

Virions were collected from three different sources, the culture supernatant fluid only, from freeze thawed infected cells only, and from a combination of both. Virions and dense bodies isolated from infected HEF and culture supernatant fluid (Section 2.2.3.) were purified by glycerol tartrate gradient centrifugation (Section 2.2.4.). Two light scattering bands were resolved, an upper light opalescent band containing enveloped virions and a lower diffuse band containing dense bodies.

Virions and dense bodies isolated from infected cells only (intracellular virus) or supernatant fluid only (extracellular virus) and virions and dense bodies isolated from infected cells and supernatant fluids (total virus), were solubilised directly in SDS PAGE reducing sample buffer (Section 2.4.3), fractionated on 10%-20% reducing SDS gels and the proteins stained with silver nitrate (Section 2.5) (Figure 15 ; Table IX).

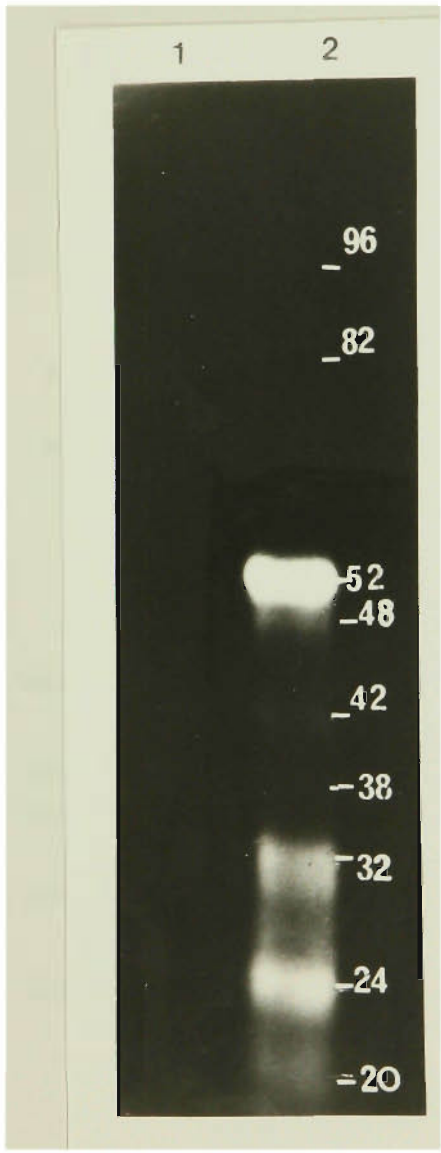


Figure. 14. Inverse image of autoradiograph of 10% SDS PAGE analysis of plasma membranes isolated from HCMV-infected and -infected cells by FMA extraction and immunoprecipitated with rabbit antisera raised against whole virions (lanes 1 and 2 respectively). Molecular weights ($X10^3$) of plasma membrane antigens of infected cells are indicated.

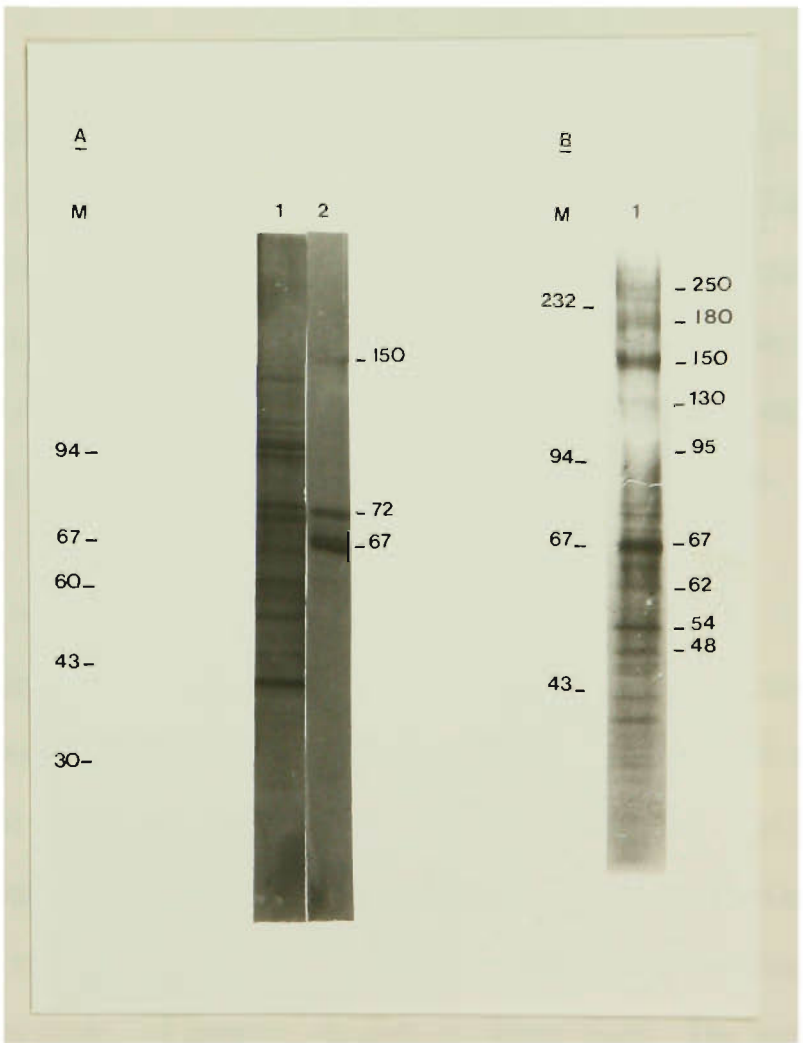


Figure 15. 10%-20% gradient SDS PAGE analysis and visualisation with silver nitrate staining of: HCMV dense bodies (A, lane 1), intracellular virus (A, lane 2) and total virus isolated from infected cells and supernatant fluid 96 hrs post infection (B, lane 1).

Proteins of M.W. 67, 72 and 150 kDa were detected for the intracellular virus sample represent the major protein constituents. However, minor protein constituents were not detected. Several more proteins were detected in extracellular virions and dense bodies isolated from supernatant fluids and infected cells (Figure 15B and 15A, lane 1). Proteins of M.W. 48, 54, 62 67, 95 130, 150, 180,and 250 kDa were detected in extracellular virions. Dense bodies, contained proteins of M.W. 38, 43, 54, 62, 67, 70, 72, 94 ,110 and 130 kDa.

Since it has been reported that immunogenic proteins of HCMV are located on the virion envelope, the isolation of these proteins was also undertaken. It has previously been suggested that HCMV virions contain two envelopes, an osmotically stable envelope which is closely attached to the nucleocapsid, and a loose-fitting osmotically fragile outer envelope. The latter envelope is said to be acquired as the virions leave the cell and can be extracted by osmotic shock of extracellular virions (Farrar and Oram, 1986).

Enveloped virions were fractionated into the following components: (i) the outer osmotically fragile envelope ; (ii) the Triton X-100 soluble envelope fraction ; and (iii) the Triton X-100 insoluble fraction (Section 2.3.2). Dense bodies were treated with Triton X-100 and the soluble fraction analysed. When the enveloped virus preparations were treated with water most of the outer envelope material was removed. Centrifugation through a glycerol tartrate gradient allowed for the separation of the outer envelope fraction in the upper band and residual virus in the lower band. The outer envelope fraction was washed in TBS by centrifugation and pelleting and used directly for analysis by reducing SDS PAGE (Figure 16, lane 4;Table VII, F). The results presented in Table VII are a summary of three isolations and SDS PAGE analysis. Three proteins of M.W. 54, 62 and 67 kDa were detected. The residual virus recovered from the lower band was used for the further isolation of osmotically stable virion envelopes and extracted with detergent. Treatment of virions and dense bodies with Triton X-100 allowed for the isolation of a Triton X-100 soluble fraction containing envelope proteins and the Triton

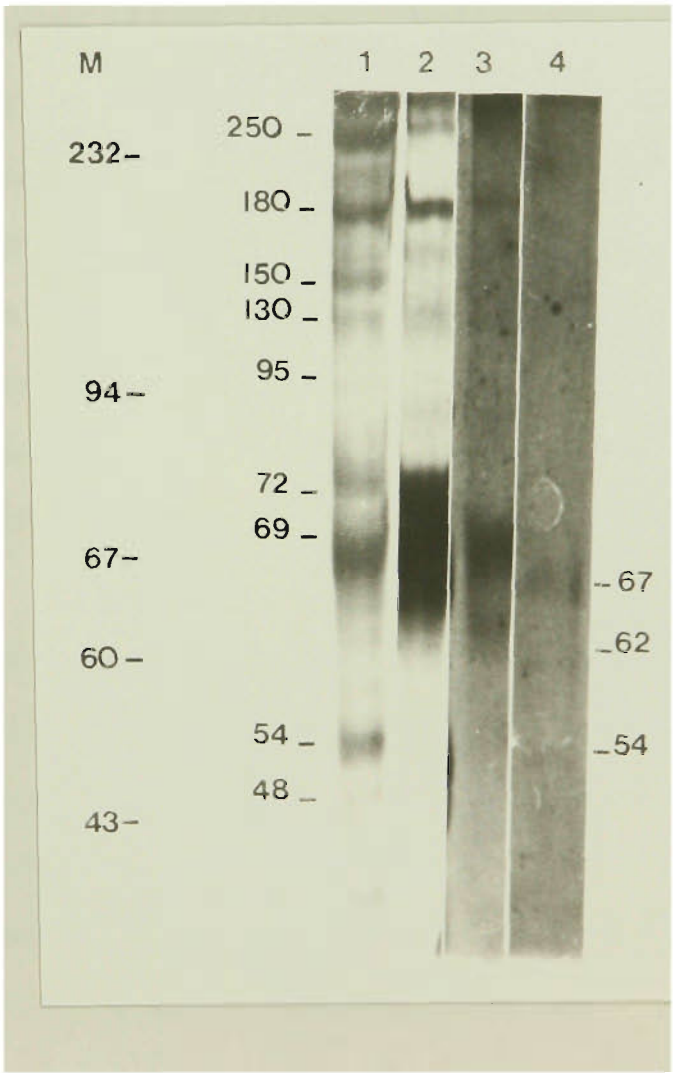
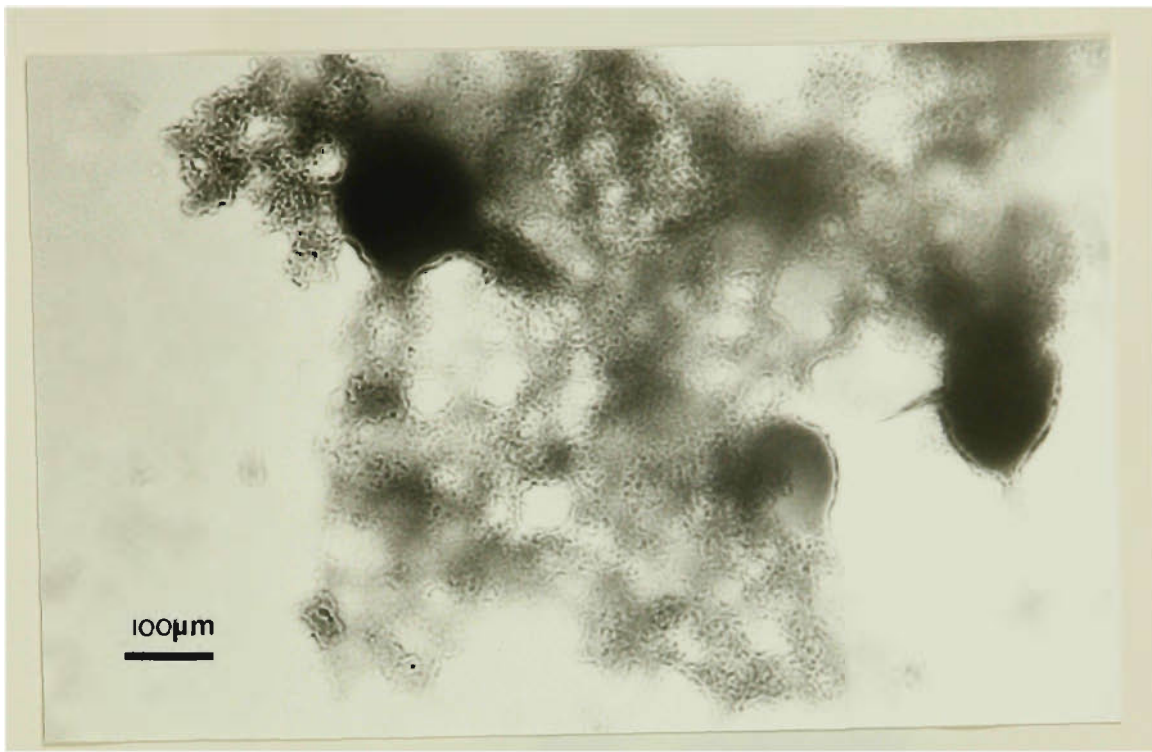


Figure 16 10% SDS PAGE analysis and visualisation with silver nitrate staining of HCMV dense bodies isolated by glycerol tartrate gradient centrifugation (lane 1); the insoluble fraction from virions treated with Triton X-100 (lane 2); virion envelope fraction after solubilisation with Triton X-100 (lane 3) ; virion outer envelope fragments prepared from osmotic shock (lane 4). Molecular weights ($X10^3$) of markers (M) and proteins are indicated.

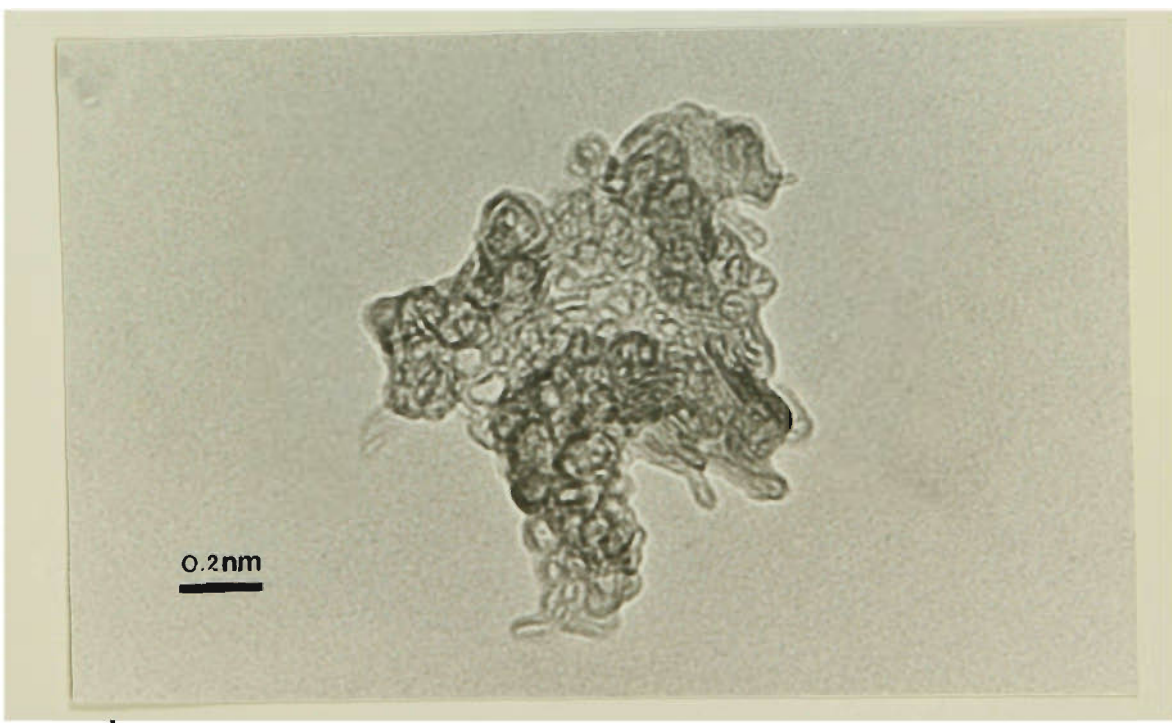
X-100 insoluble fraction containing nucleocapsid proteins. The Triton-X soluble fractions were treated with SM B10 beads, before analysis by reducing SDS PAGE and silver nitrate staining (Figure 16, lanes 1 and 3). The Triton X-100 insoluble fraction was treated with SDS PAGE reducing sample buffer and analysed directly (Figure 16, lane 2). Recovery of Triton X-100 solubilised envelopes from dense bodies (lane 1) resulted in several proteins, these being of M.W. 48 , 54 , 69 , 72 , 95 , 130 , 150 , 180 and 250 kDa. Most of these proteins represent virion envelope proteins, as shown in Figure 14B. The virus matrix protein of 64-70 kDa is noticeably absent in dense body preparations. Proteins isolated from the Triton X-100 treated virion fraction (lane 3) after the removal of osmotically fragile proteins were of M.W. 62, 65-68 and 180 kDa. Virion envelopes removed either by osmotic shock or extracted with Triton X-100 were examined by scanning electron microscopy to determine the purity of each fraction (Figure 17).

To improve detection of purified virion envelope proteins, proteins were labelled with ^{125}I, using the chloramine T-procedure (Section 2.11) and analysed by 10% reducing SDS PAGE and autoradiography (Section 2.8). Virions were purified using glycerol tartrate gradients and the envelope extracted with either Triton X-100, sodium deoxycholate or osmotic shock. Envelope constituents were labelled with ^{125}I and analysed by 10% SDS PAGE and autoradiography, Table VIII.

No differences in labelled envelope protein was detected for the Triton X-100 and sodium deoxycholate extracted envelopes from either extracellular virus or intracellular virus (Figure 18). All fractions contained an envelope protein of M.W. 52-54 kDa. Additional proteins of M.W. 62, 43, 32 and 28 kDa were identified in the Triton X-100 extracted intracellular virus sample.



17a



17b

Figure 17a.

and 17b. Elecron micrographs of virion envelopes obtained from Triton X - 100 treatment of virions and dense bodies (a) and osmotic shock (b).

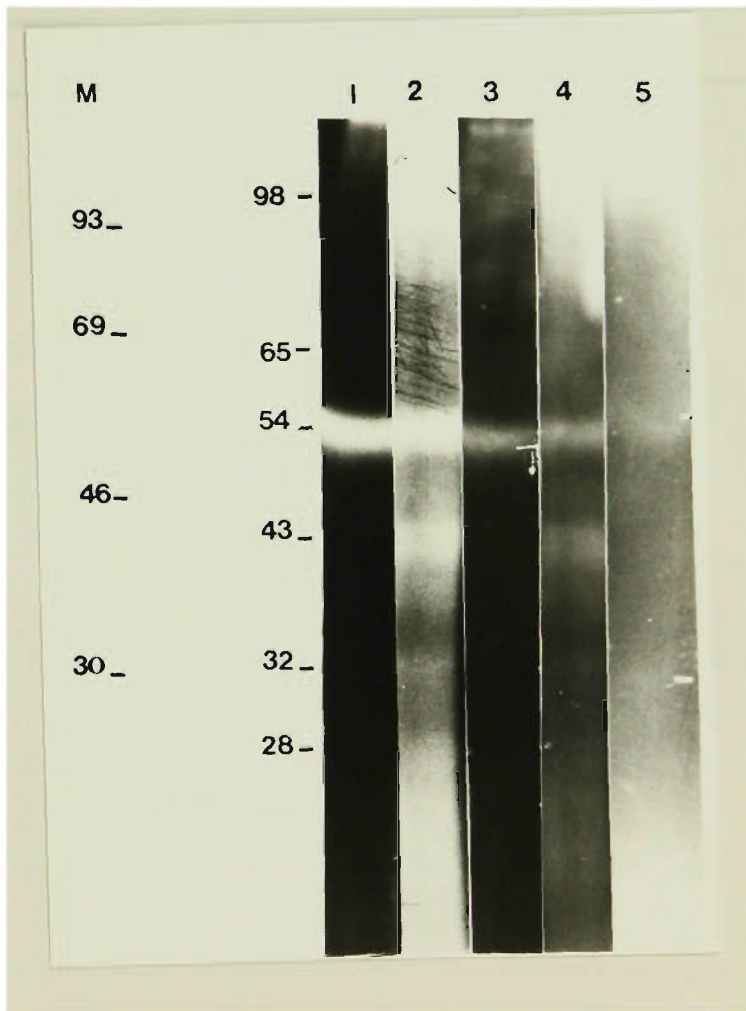


Figure 18. Inverse image of autoradiograph of 10% SDS PAGE analysis of ^{125}I- labelled total virus solubilised with Triton X-100 (lane 2) and sodium deoxycholate (lane 3) after the removal of the outer osmotically fragile envelope (lane 5). Lanes 1 and 4 represent virion envelope fractions isolated from extracellular virus solubilised with Triton X-100 and sodium deoxycholate respectively. Molecular weights ($X10^3$) of ^{14}C labelled markers (M) and virus envelope proteins are indicated.

Table VII.

Characterization of Proteins from HCMV Virions, Dense Bodies and Virion Envelopes.

A	B	C	D	E	F
250	250	-	250	-	-
180	180	-	180	180	-
-	-	-	160	-	-
150	150	150	-	-	-
130	130	-	130	-	-
95	95	-	-	-	-
-	72	72	-	-	-
67	66-69	67	65-70	65-68	67
62	64	-	-	62-64	62
-	60	-	-	-	-
-	58	-	-	-	-
54	50-54	-	-	-	54
48	48	-	-	-	-
46	46	-	-	-	-
45	-	-	-	-	-
43	-	-	-	-	-
40	-	-	-	-	-
38	-	-	-	-	-
28	-	-	-	-	
26	-	-	-	-	-
20	-	-	-	-	-

Molecular weights ($X10^3$) of virion proteins identified from reduing SDS PAGE and silver nitrate staining of the total virus fraction, (A), dense bodies, (B), the intracellular virus,(C), the Triton X-100 insoluble fraction, (D), the Triton X- 100 soluble fraction (E) and the water soluble fration (F).

Table VIII.

^{125}I labelled Virion and Virion Envelope Proteins.

A	B	C	D	E
-	98	-	-	-
-	65	-	-	-
54	54	54	54	54
-	-	48	-	-
-	43	-	43	-
-	32	-	-	-
-	28	28	-	-
20	-	22	-	-
-	-	18	-	18
-	16	15	-	14
-	12	-	-	-

^{125} I labelled proteins of the extracellular virus fraction (A), total virus solubilized with Triton-X-100, (B), total virus solubilized with sodium deoxycholate (C), extracellular virus solubilized with sodium deoxycholate, (D) and viron envelope proteins obtained from osmotic shock (E) characterized by reducing SDS PSGE and autoradiography. The molecular weights (X 10^3) of labelled proteins are listed above.

3.3.3. Reactivity of Virion Proteins and Virion Envelope Proteins with Antisera Raised Against HCMV Induced Plasma Membrane Proteins.

Since it had been established that HCMV induced plasma membrane proteins are antigenically related to virion proteins (Section 3.3.1.) the reactivity of a variety of antisera with ^{125}I labelled virions and proteins of detergent extracted and osmotically fragile virion envelopes was assessed. The antisera used were raised in rabbits against HCMV-induced plasma membrane proteins of 54, 62 and 94 kDa. Mab α52, specific for envelope glycoprotein gp 52 (Farrar and Greenaway, 1986) was also used (Figures 19 and 20; Table IX). The results reported here are from experiments performed in duplicate.

Antisera (IgG), prepared according to the procedures given in Section 2.6.1. raised against plasma membrane protein 94 kDa, immunoprecipitated virion envelope proteins of M.W. 54-58, 62 kDa and virion proteins of 54-58, 62, 68, 94, 130, 150 and 250 kDa (Figure 19). The antisera did not react with proteins of the envelope fraction obtained from osmotic shock. However, Mab α52 reacted with proteins of 52, 60 and 68 kDa of the latter fraction.(Figure 19).

Anti-54 kDa IgG and anti-62 kDa IgG immunoprecipitated virus envelope proteins of molecular weight 54 kDa from both theTriton X-100 extracted envelope fraction and the osmotically fragile envelope fraction (Figure 20). A protein of 62 kDa was also detected with the anti-62 kDa IgG in the Triton X-100 fraction. Thus two of the three antisera raised against HCMV-induced plasma membrane proteins reacted with proteins from the outer osmotically fragile envelope of the virion.

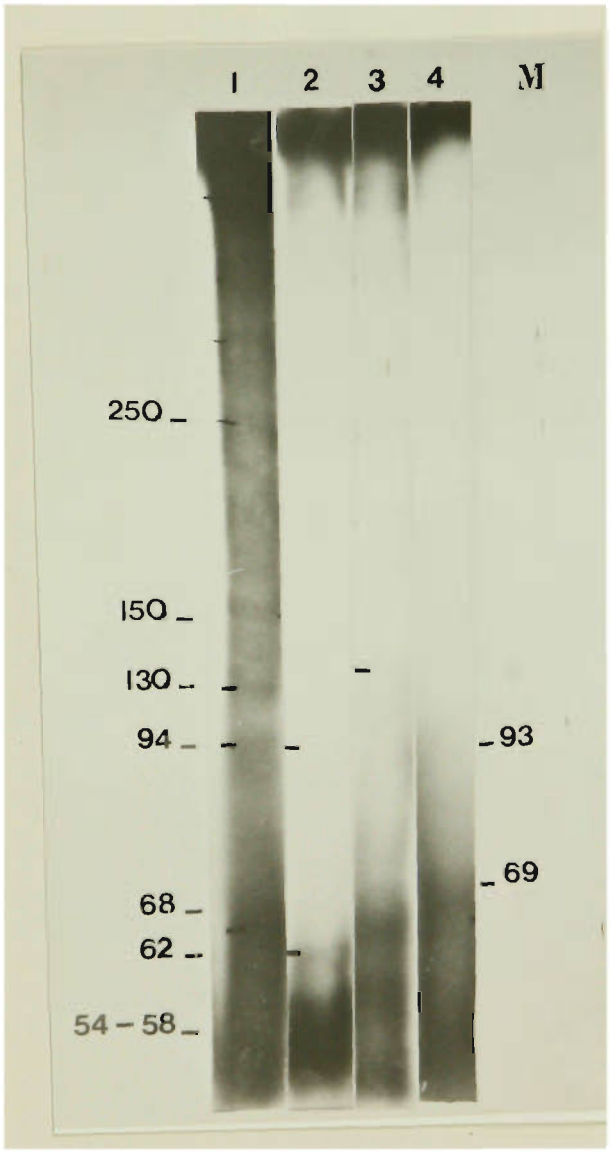


Figure 19. Autoradiograph of ^{125}I labelled immunoprecipitates of virions and virion envelopes analysed by 10% SDS PAGE. Triton X-100 solubilised extracellular virus (lane 4) and total virus (lane 1) reacted with anti-94 kDa IgG. Purified virion envelopes solubilised with Triton X-100 were reacted with anti-94 kDa IgG (lane 2) and murine monoclonal antibody α52 (lane 3). Molecular weights ($X10^3$) of virion and virion envelope proteins are indicated.

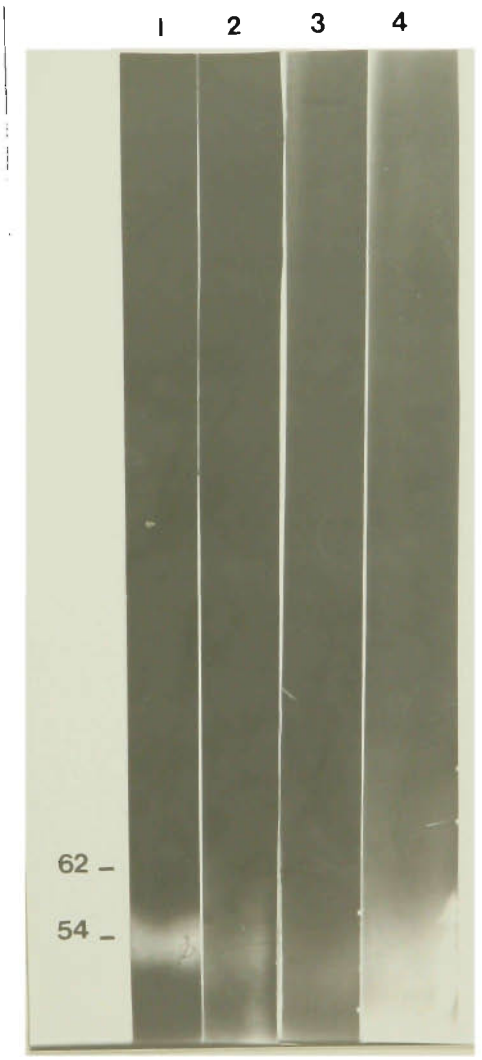


Figure 20. Inverse image of autoradiograph of ^{125} I labelled immunoprecipitates of virion envelope fragments extracted sequentially by osmotic shock and then Triton X-100 solubilisation. Osmotic shock virion envelope fragments were reacted with rabbit IgG raised against plasma membrane proteins of 62 kDa and 54 kDa from infected cells (lanes 1 and 3 respectively). Triton X-100 solubilised virion envelopes were also reacted with anti-62 kDa and anti-54 kDa sera (Lanes 2 and 4 respectively).

Table IX.

Proteins Immunoprecipitated from ^{125}I labelled virion and virion envelope proteins with antisera raised against plasma membrane proteins of M.W. 54, 62, and 94 kDa and Mab α52.

	A	B	C	D	E
Total Virus	250	-	-	-	-
	150	-	-	-	-
	130	-	-	-	-
	94	-	-	84	-
	68	68	68	68	-
	62	-	-	-	60
	54-58	54	54	-	52-54
	-	-	22	-	-
Extracellular Virus	-	-	-	ND	82
	68	68	68		68
	-	-	-		60
	54-58	54	54-58		52-54
	-	-	-		48
Triton-X-100 SolubleFraction	-	-	-	120	-
	-	-	-	-	82
	-	-	-	68	-
	62	62	-	64	60
	54-58	54-58	54-58	54-58	52-54
	-	-	-	52	-
Virion envelopes (osmotic shock)	-	54-58	54-58	ND	ND

Proteins from the total virus fraction , extracellular virus fraction, virion Triton X-100 soluable fraction and virion envelopes obtained from osmotic shock, immunoprecipitated with anti-94 kDa sera, (A), anti-62 kDa sera (B), anti- 54 kDa sera (C), Mab α52 (D) and convalescent HCMV sera (E) and analysed by reducing SDS PAGE and autoradiography. Molecular weights (x 10^3) of proteins are listed above. ND:- not done.

3.4. Reactivity of HCMV-Induced Plasma Membrane Proteins with Antisera Raised Against Plasma Membrane Proteins.

The presence of HCMV-induced protein complexes within the plasma membranes of infected cells was investigated. Plasma membranes isolated by the two-phase polymer systems from uninfected and HCMV-infected HEF, 72h after infection (Section 2.3.1), and cell lysates from similar cells, were each solubilized in 1% NP40 and 1% sodium deoxycholate (Section 2.4.2), and immunocomplexed with protein A purified IgG from antisera raised against infected plasma membrane glycoproteins of M.W. 54, 62 and 94 kDa . Membrane proteins reactive with the antisera were collected by incubation of Sepharose beads in non-reducing SDS PAGE sample buffer (Sections 2.4.1 and 2.5) and loaded directly onto 5% or 7.5% linear polyacrylamide gels. To facilitate adequate separation of high molecular weight protein complexes (HMWC), the samples were electrophoresed for an additional hour after the marker dye had reached the bottom of the gel (Section 2.13). The plasma membrane protein complexes and cell protein complexes reactive with anti-54 IgG, anti-62 IgG and anti-94 kDa IgG, are shown in Figures 21, 23 and 25 and summarised in Table X.

A diffuse HMWC of 130-150 kDa reactive with anti-54K IgG was obtained for plasma membranes from infected and uninfected cells, but a HMWC of >150 kDa was detected only in the total cell protein fraction from infected cells (Figure 21). Three distinct HMWC's of 130, 140 and 150 kDa were detected with anti-62 kDa IgG and anti-94 kDa IgG for plasma membranes isolated from infected cells. The same HMWC's were also isolated from the total cell protein of infected cells using anti-62 kDa IgG and anti-94 kDa IgG (Figures 23 and 25).

Table X.

Labelled Proteins Isolated by Immunoprecipitation of Plasma Membrane Proteins and Total Cell Lysates from Infected and Uninfected Fibroblasts with Antisera and Analysed by Non-Reducing and Reducing SDS Electrophoresis.

HMWC's

	Infected		Uninfected	
	m	l	m	l
Anti-54 kDa	130-150	150-200	130-150	-
	-	55	-	-
Anti-62 kDa	150	130-150	-	150
	140	80-94		140
	130	55	-	130
	94		-	94
	55			
Anti 94 kDa	150	130-150	-	150
	140	80-94		140
	130	55	-	130
	94	-	-	80-94
	55			
Reduced Proteins				
Anti-54 kDa	130	ND	-	ND
	92-94			
	69			
	54-58			
Anti - 62 kDa	130	130	-	130
	94	120	-	-
	69	110	-	-
		92	-	-
	54-58	54-58		
Anti-94 kDa	130	130	ND	130
	110	120		-
	92	110		-
	69	92		92
	54-58	54-58		54-58
Mab 28-2	130	ND	-	ND
	94		-	
	54-58		-	
Mab 7-17	130	130	-	-
	94	94	-	-
	-	68	-	-
	54-58	54-58	-	-

Metabolically labelled proteins from plasma membranes (m) and whole cell lysates (l) of HCMV infected and uninfected fibroblasts immunoprecipitated with anti- 54 kDa IgG, anti- 62 kDa IgG and anti- 94 kDa IgG, and analysed by reducing and non reducing SDS PAGE and autoradiography. Molecular weights (x 10^3) of proteins are listed above. ND designates :- not determined.

The HMWC's of the infected cells indicated a much higher affinity for the antisera than the HMWC's immunoprecipitated from the uninfected cells. No labelled HMWC's were detected for plasma membranes from uninfected cells immunoprecipitaed with anti- 62 kDa IgG and anti- 94 kDa IgG. Proteins of M.W. 94 and 55 kDa were identified in both the plasma-membrane and whole cell lysate fractions with anti-62 IgG and anti-94 kDa IgG. In order to establish the protein sub-unit composition that go to make up the HMWC's, the labelled HCMW's were excised from the dried gels and treated with reducing SDS PAGE sample buffer containing 2% mercaptoethanol (2-ME) (Section 2.4.5). If no labelled HMWC's could be detected on the auotoradiographs, in this instance plasma membrane proteins from uninfected cells immunoprecipitated with anti-62 kDa IgG and anti- 94 kDa IgG, the protein bands were excised from the gels in the relative position of the HMWC's of the labelled HMWC's from infected cells. These control experiments were carried out in order to determine whether any host plasma membrane proteins in normal cells, not synthesized at the time of labelling or synthesized in relatively low amounts, not detectable on the autoradiograph due to the relatively short time period of exposure of the X-ray film, did react with the antisera. The proteins were re-electrophoresed on 10% linear SDS gels. Figures 22, 24, 26, 27, 28 and 29 provide typical profiles for the protein composition, under denaturing conditions of the HMWC's of plasma membranes from infected cells reactive with anti-54 kDa IgG, anti-62 kDa IgG and anti-94 kDa IgG. The results are summarized in Table XI.

Both the HMWC of 130-150 kDa from plasma membranes and HMWC of > 150 kDa from cell lysates of infected cells, immunoprecipitated by anti-54 kDa IgG, fractionated into four distinct protein bands of 150, 130, 92-94, 55 kDa and a possible band at < 55 kDa (Figure 22). The 55 kDa protein was the most abundant. In contrast, the corresponding HMWC of 130-150 kDa from plasma membrane preparations and HMWC of > 150 kDa from cell lysates of uninfected cells contained a protein of slightly <55 kDa,

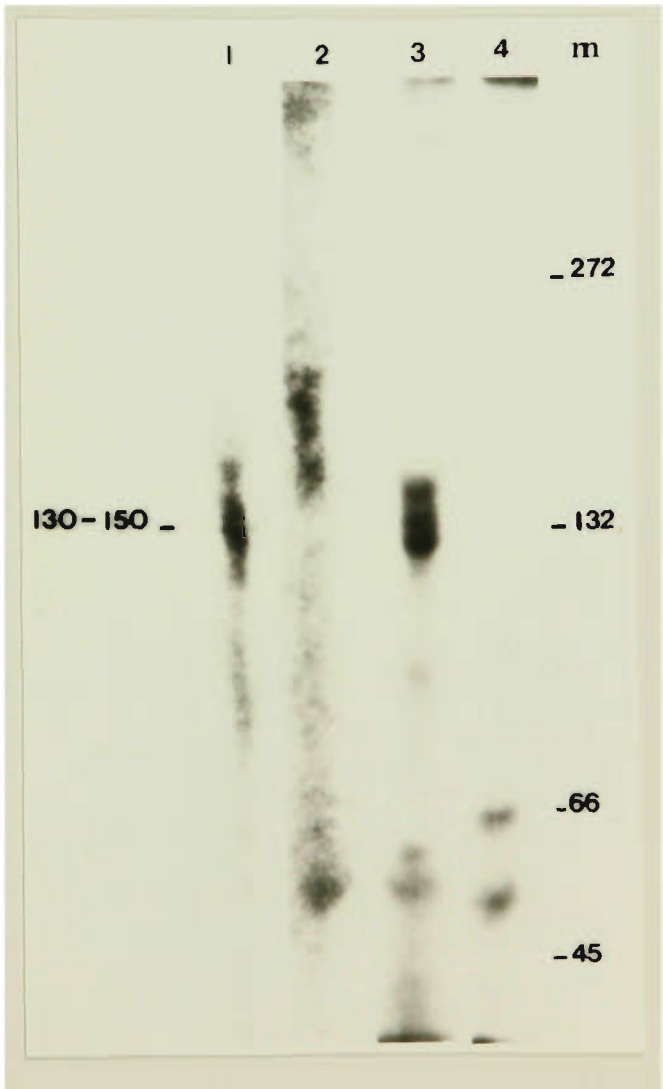


Figure 21. Immunoprecipitation with anti-54 kDa IgG of metabolically labelled proteins from cell lysates and plasma membrane extracted 72 hrs post infection. Autoradiograph of linear 7.5% non-reducing SDS PAGE of proteins from plasma membranes and cell lysates from HCMV infected (lanes 1 and 2 respectively) and uninfected cells (lanes 3 and 4 respectively). Molecular weights ($X10^3$) of protein complexes and non-reduced markers (M) are indicated.

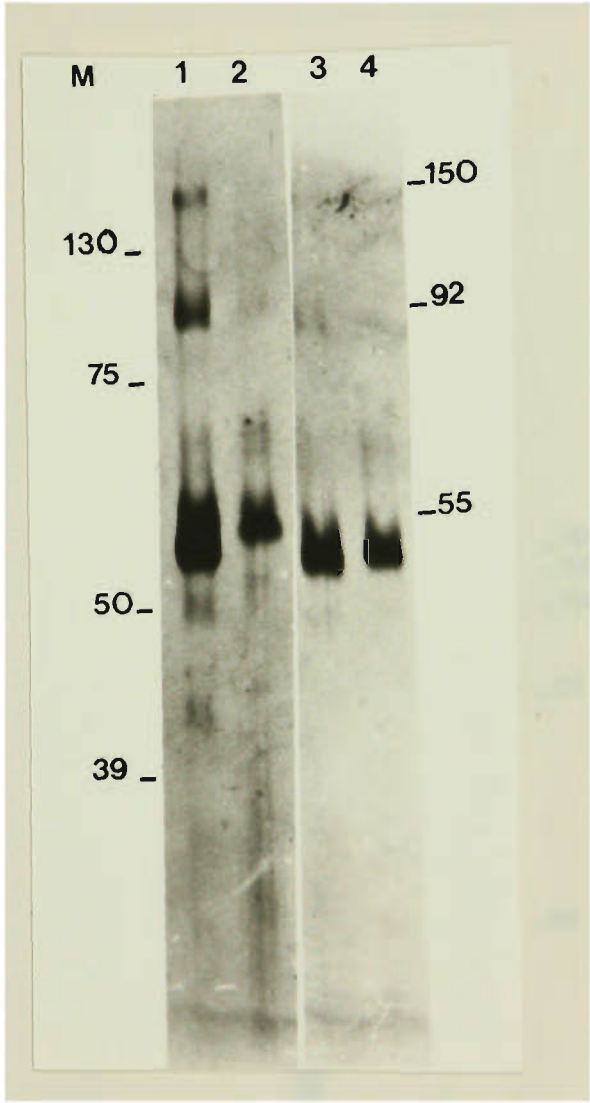


Figure 22. Re-electrophoresis in 10% SDS gels, of high molecular weight disulphide linked protein complexes (HMWC), from cell lysates and plasma membranes of infected cells immunoprecipitated with anti-54 kDa IgG and visualised with silver nitrate after reducing PAGE.

HMWC of 130-150 kDa from plasma membranes of infected and uninfected fibroblasts (lanes 1 and 3 respectively) and cell lysates of infected and uninfected fibroblasts (lanes 2 and 4 respectively) were immunoprecipitated. Molecular weights ($X10^3$) of proteins and markers (M) are indicated.

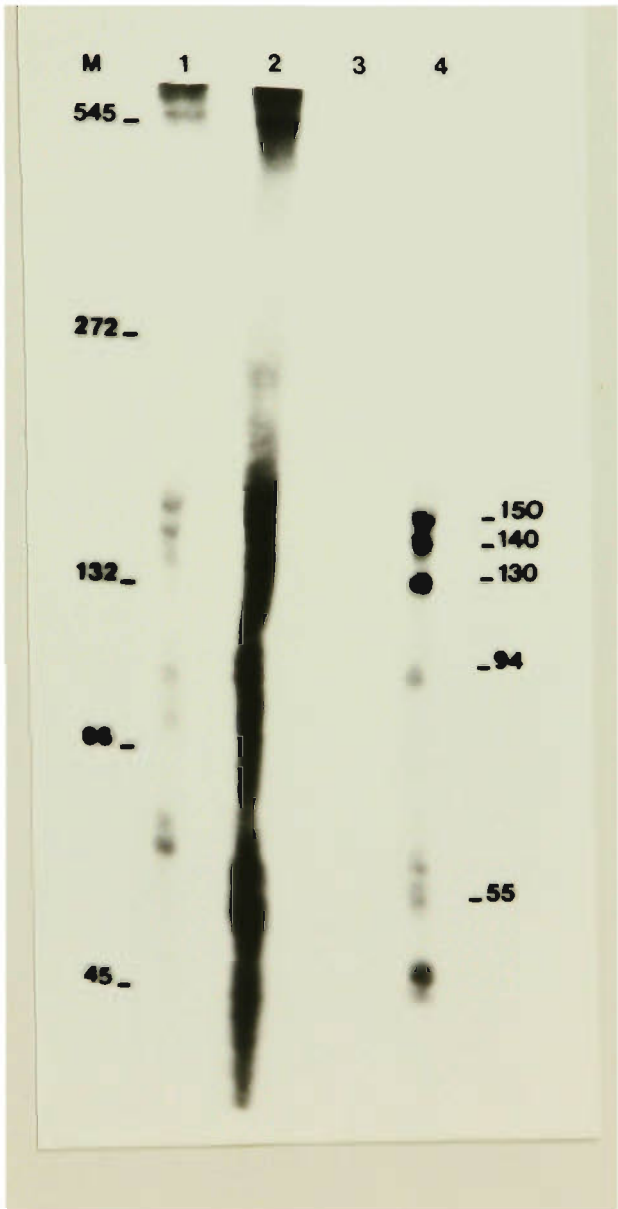


Figure 23 Immunoprecipitation with anti-62 kDa IgG of metabolically labelled proteins from cell lysates and plasma membrane extracts isolated 72 hrs post infection Autoradiograph of 7.5% non-reducing SDS PAGE of proteins from uninfected and HCMV infected cell (lanes 1 and 2 respectively) and plasma membranes from uninfected and infected fibroblasts (lanes 3 and 4 respectively). Molecular weights ($X10^3$) of protein complexes and markers (M) are indicated.

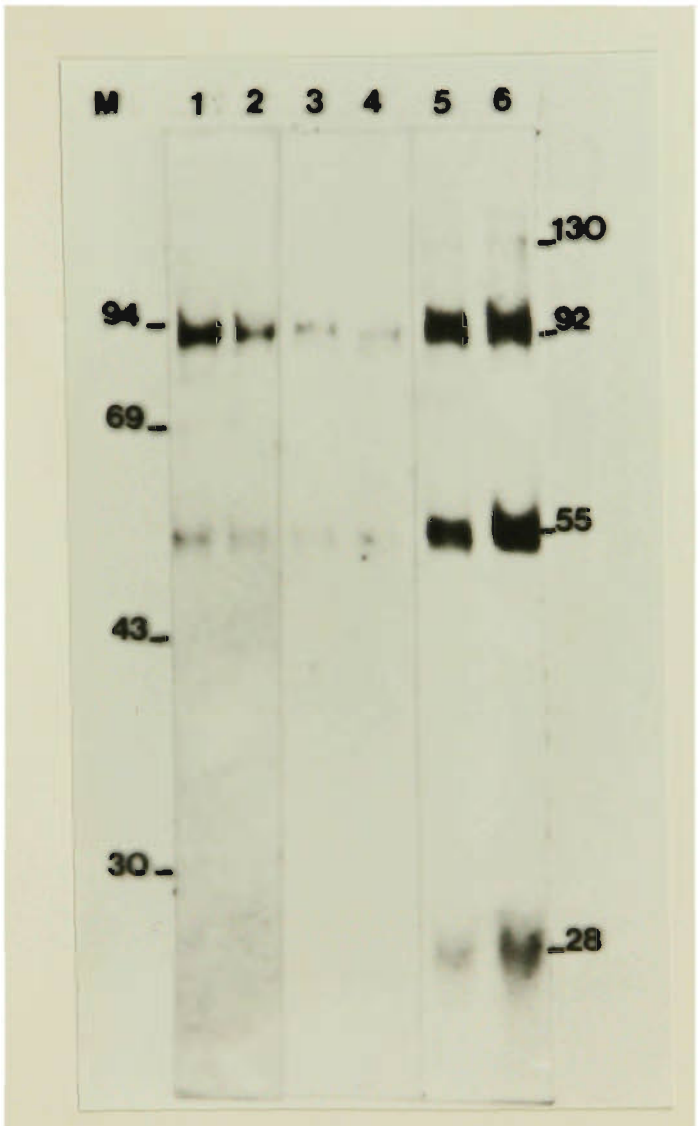


Figure 24. Re-electrophoresis in 10% SDS gels of HMWC's from non-reducing gels of infected cell extracts and plasma membranes immunoprecipitatedwith anti-62 kDa IgG.

Silver stain of reduced HMWC's of 150 kDa from plasma membranes isolated from uninfected and infected cells (lanes 4 and 6 respectively) and proteins from uninfected and infected cell extracts (lanes 3 and 5 respectively). Reduced proteins of HMWC 140 kDa of infected cell extracts and plasma membranes are shown in lanes 1 and 2 respectively. Molecular weights ($X10^3$) of markers (M) and reduced proteins are indicated.

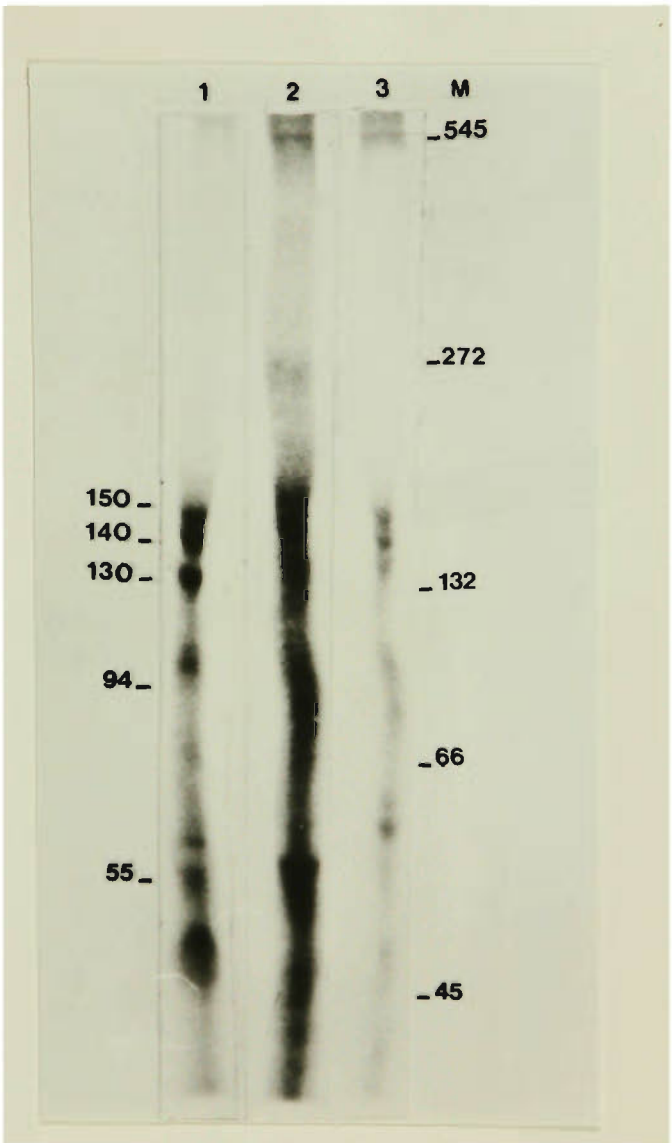


Figure 25 Immunoprecipitation with anti-94 kDa IgG of metabolically labelled proteins from cell lysates and plasma membrane extracts isolated 72 hrs post infection.

Autoradiograph of 7.5% non-reducing SDS PAGE of HCMV infected and uninfected cell extracts (lanes 2 and 3 respectively) and plasma membrane proteins from infected fibroblasts (lane 1). Molecular weights of HMWC's ($X10^3$) and non-reduced markers (M) are indicated.

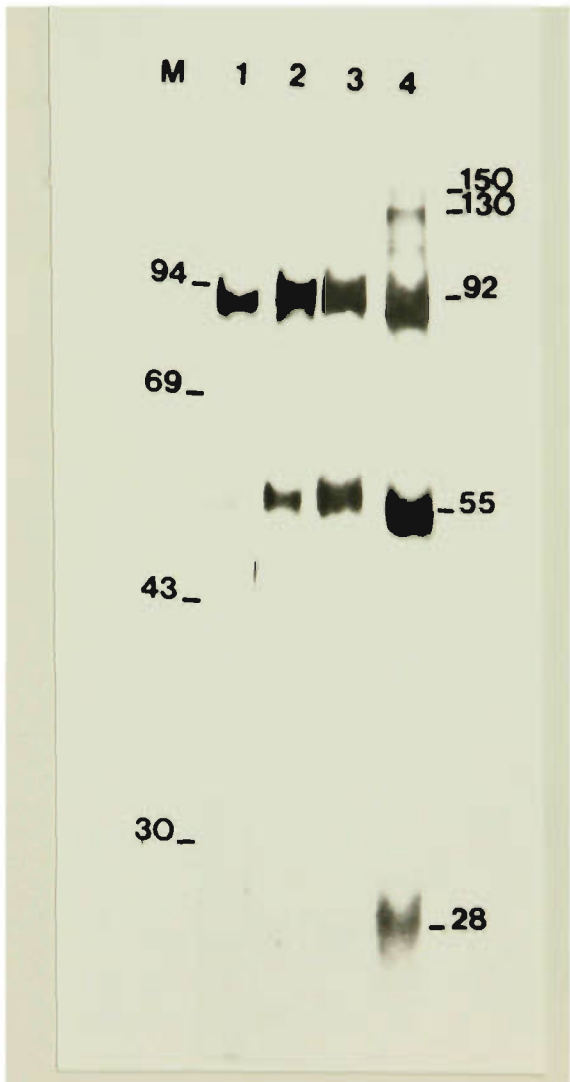


Figure 26. Re-electrophoresis in 10% SDS gels of HMWC's from non-reducing gels of cell lysates and plasma membranes from infected cells only, immunoprecipitated with anti-94 kDa IgG. Silver stain of reduced HMWC's of 140 and 150 kDa from infected cell lysates and plasma membranes from infected fibroblasts (lanes 1 and 2, and lanes 3 and 4 respectively). No proteins were detected by re-electrophoresis of complexes isolated from uninfected cell extracts (data not shown). Molecular weights of proteins ($X10^3$) and markers (M) are given.

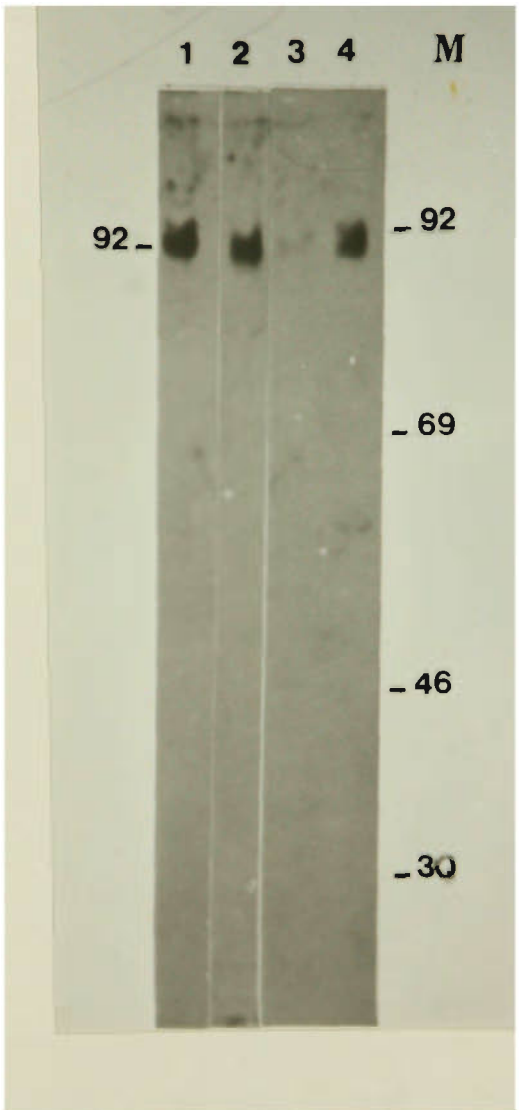


Figure 27 Re-electrophoresis on 7.5%-15% gradient SDS gels of HMWC 130 kDa isolated by immunoprecipition with anti-94 kDa or anti-62 kDa. Cell lysates and plasma membranes from infected cells reactive with anti-94 kDa (lanes 1 and 2 respectively); membranes from infected cells and cell lysates from uninfected cells reactive with anti-62 kDa (lanes 3 and 4 respectively). Molecular weights ($X10^3$) of proteins and markers (M) are given.

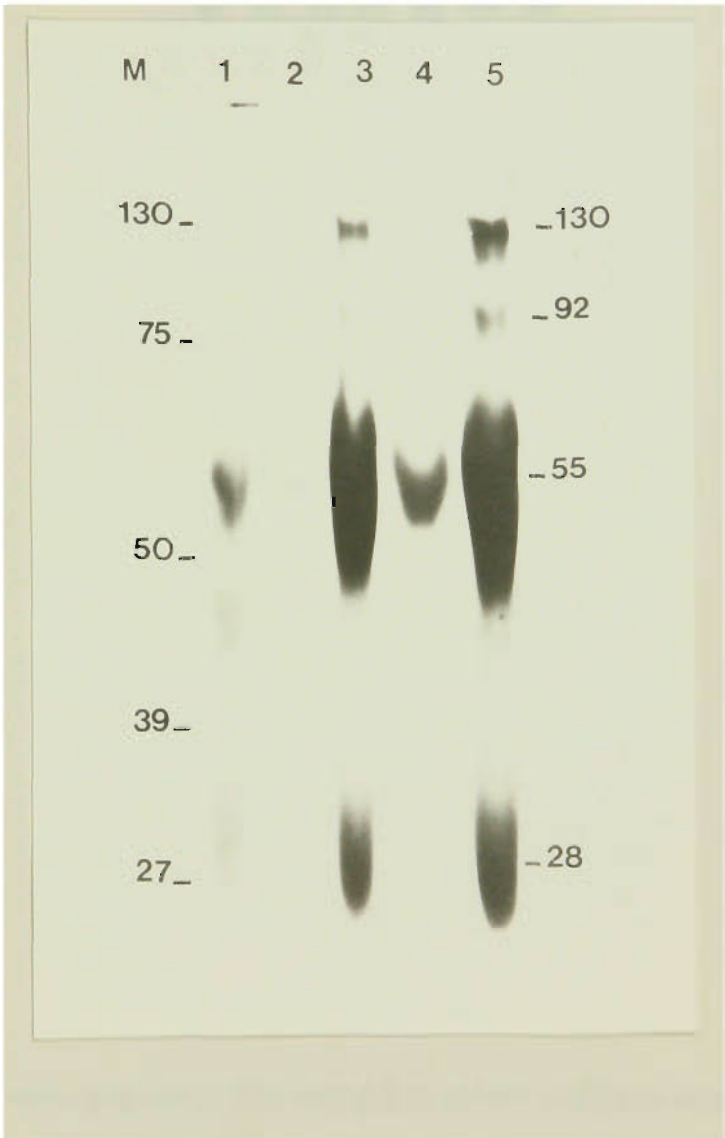


Figure 28 Re-electrophoresis with 4% 2-ME on 7.5%-15% gradient SDS gels of HMWC 150 kDa isolated in non-reducing gels of plasma membranes from infected and uninfected cells, immunoprecipitated with: serum 6PP (lanes 1 and 2 respectively), anti-62 kDa IgG (lanes 3 and 4 respectively), and anti-94 kDa IgG (lane 5). No HMWC was re - electrophoresed for plasma membranes from uninfected cells immunoprecipitated with anti- 94 kDa IgG. Molecular weights ($X10^3$) of membrane proteins and markers (M) are indicated.

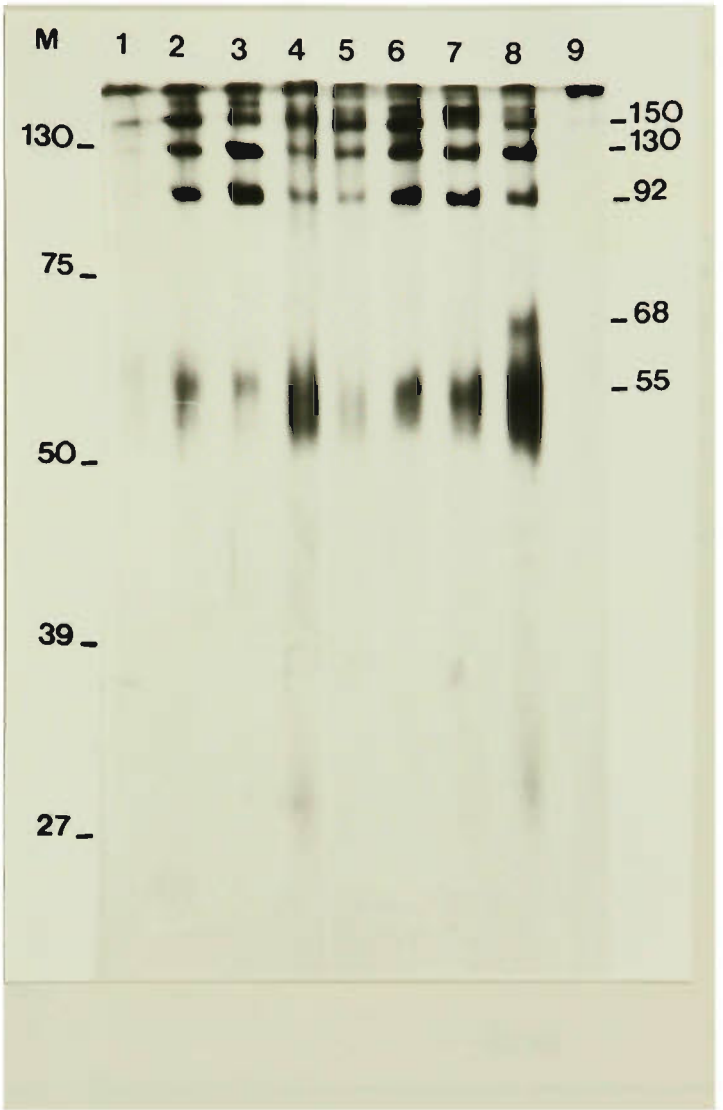


Figure 29 Re-electrophoresis on 10% SDS gels of HMWC > 500 kDa isolated on non-reducing gels from cell extracts and plasma membranes immunoprecipitated with anti-62 kDa, anti-94 kDa and guinea pig serum 6PP. HMWC > 500 kDa reactive with anti-62 kDa from uninfected cell extracts and plasma membranes (lanes 2 and 3 respectively) and infected cell extracts and plasma membrane (lanes 6 and 7 respectively). Uninfected and infected cell extracts reactive with anti-94 kDa IgG (lanes 1 and 5 respectively) and plasma membranes from infected cell reacted with the same antisera (lane 8) Membranes from uninfected and infected cells precipitated with serum 6PP (lanes 9 and 4 respectively). Molecular weights ($X10^3$) of proteins and markers (M) are given.

Table XI.

Reduction and Re-electrophoresis of HMWC's Immunoprecipitated from HCMV Infected cell Lysates and Plasma Membranes with Antisera.

HMWC	Infected		Uninfected	
	m	l	m	l
Anti-54 kDa IgG				
130-150	150	-	-	-
	130	-	-	-
	92-94	94	92-94	-
	64	64	-	-
	55	55	< 55	< 55
Anti-62 kDa IgG				
>500	160	160	160	160
	150	150	150	150
	130	130	130	130
	92	92	92	92
	55	55	55	55
150	130	130	-	-
	92-94	92-94	92-94	92
	55	55	55	55
	28	28	-	-
140	92-94	-	-	-
	55	-	-	-
130	-	-	-	92-94
Anti-94 kDa IgG				
>500	160	160	ND	-
	150	150		150
	-	130		130
	92-94	92-94		92
	55	55		55
150	150	-	ND	-
	130	-		-
	92-94	92-94		-
	55	55		-
	28	28		-
140	94	94	ND	-
	55	55		-
130	92-94	92-94	ND	-

Molecular weights (x 103) of proteins obtained from the reduction and re-electrophoresis of HMWC immunoprecipitated from HCMV infected cell lysates and plasma membranes with antisera raised against plasma membrane proteins of M.W. 54, 62 and 94 kDa.

m - plasma membranes ; l - cell lysates.; ND - not determined.

and no proteins of M.W. 130 or 92-94 K Da These results indicate a difference in the sub-unit composition of the HMWC's from plasma membrane and cell lysate preparations from HCMV infected and uninfected cells immunoprecipitated with anti- 54 kDa IgG .

The HMWC of 150 kDa from both the plasma membrane preparations and total cell lysates from infected cells reactive with anti-62 IgG and 94 kDa IgG contained abundant protein sub-units of 92-94, 55 and 28 kDa (Figures 24 and 26, respectively). Two minor bands at 130 and 150 kDa were also detectable. These could represent an incomplete dissociation of disulphide-linked proteins of the HMWC 150 kDa or the presence of two structurally unrelated sub-unit proteins that make up HMWC 150 kDa.

Upon reduction of the gel slices containing possible HMWC's of 150 and 140 kDa isolated from uninfected cell lysates with anti-94 kDa IgG no proteins could be detected with silver staining, whilst the HMWC of 150 kDa isolated from plasma membrane fraction and cell lysates from uninfected cells, with anti-62 kDa (Figure 23, lanes 3 and 4, respectively) yielded minor protein bands at 92 and 55 kDa and no protein band at 28 kDa when re-electrophoresed and silver stained (Figure 24).

No proteins were detected after analysis under reducing conditions of the HMWC 140 kDa isolated from either plasma membranes or cell lysates from uninfected cells with anti-62 kDa IgG. Proteins of 94 and 55 kDa were detected by reducing SDS PAGE analysis of HMWC's of 140 kDa isolated from infected cell plasma membrane preparation and infected cell lysates with anti-62 IgG and anti-94 kDa IgG (Figures 24 and 26, respectively). The minor protein bands detected at 92 and 55 kDa after the reduction of HMWC of 150 kDa of uninfected cell lysates and plasma membrane preparations, immunoprecipitated with anti-62 kDa and anti-94 kDa, could represent the same or different host proteins of similar molecular weights to those identified in the virus infected cells. That no proteins were detected when the HMWC of 140 kDa from uninfected cells was reduced indicates that this complex and its consituent sub-unit proteins could be

unique to virus infected cells, or that the amount of protein isolated from the HMWC was insufficient to allow for detection of proteins after re-electrophoresis.

Reduction of the HMWC of 130 kDa immunoprecipitated with the anti- 94 kDa IgG from infected cells and plasma membranes resulted in only one protein of 92 kDa (Figure 27). A similar result was obtained when the HMWC of 130 kDa for uninfected cell lysates reactive with anti- 62 kDa IgG was analysed by reducing PAGE and silver staining. A minor band at 92 kDa was detected after reducing gel analysis of the 130 kDa complex reactive with anti- 62 kDa IgG for the plasma membranes of infected cells . These results probably represent the incomplete reduction of the HMWC 130 kDa. As such more stringent reducing conditions using 4% 2ME were employed to reduce proteins of the HMWC's, this treatment being undertaken in the same gel slice after the former treatment with 2% 2ME.

Reduction of the HMWC 150 kDa isolated from plasma membranes of infected cells with anti-62 IgG and anti-94 IgG with 4% 2ME confirms the results expressed in Figures 24 and 26 . The 150 kDa complex reactive with both antisera contains proteins of 130, 92, 55 and 28 kDa (Figure 28). The only cross reacting host protein is of M.W. 55 kDa, indicating that the HMWC of 150 kDa of virus infected cell and plasma membrane preparations differs from the HMWC of the uninfected cells, and the the antisera is specific for a virus specified proteins

Reduction, re-electrophoresis and silver staining of the HMWC of >500 kDa isolated from infected cell plasma preparations and and total cell lysates with anti-62 IgG and anti-94 IgG gave rise to proteins of 150 , 130 , 92 , 68 and 55 kDa (Figure 29). However, the HMWC of >500 kDa from the uninfected cell plasma membrane fraction and the uninfected cell lysate fraction immunoprecipitated with anti-62 IgG and anti-94 IgG also contained reduced proteins of 150, 130, 92 and 55 kDa. However the amount of protein detected at 55 kDa, shows higher amounts of this protein and in some instances possibly

more than one protein (Figure 29, lanes 7 and 8) for reduced HMWC's of >500 kDa from infected cells than uninfected cells. It could be that HMWC >500 kDa represent a multimer of the 150 kDa complex found in normal and infected cells.

In summary a HMWC of 130-150 kDa immunoprecipitated from plasma membrane preparations of infected cells with anti-54 kDa IgG has a component unique to the infected cells. Anti-94 kDa IgG also immunoprecipitates a novel complex of 130-150 kDa from infected cells. Anti 62 kDa IgG appears to immunoprecipitate a novel 140 kDa complex in infected cells. However a host protein complex in a similar molecular weight range can be immunoprecipitated by all three antisera.

In order to determine the antigenic and structural association between protein complexes in the plasma membranes of infected cells, and to compare them to protein complexes of cell lysates, both fractions were immunoprecipitated with anti-54 IgG, anti-62 IgG and anti-94 kDa IgG and solubilised directly in reducing SDS PAGE sample buffer containing 2% 2-ME (Section 2.4.1 and 2.4.2). The proteins were analysed by SDS PAGE and fluorography (Figure 30;Table X).

Proteins of M.W. 130 , 92-94 and 54 -58 kDa were identified in plasma membrane proteins of infected cells with anti-94 kDa IgG, anti-62 kDa IgG, anti-54 kDa IgG, Mab CH28-2 and Mab 7-17, indicating that all these antisera recognise similar proteins in plasma membranes from infected cells (Figure 30 ; Table XI). The same proteins were also identified in infected cell lysates with anti-94 kDa IgG anti-62 kDa IgG and Mab 7-17. An addtional protein of <54 kDa was also detected in infected cell lysates immunoprecipitation with anti-62 kDa and anti-94 kDa (Figure 30, lanes 2 and 4). A protein of ~ 86-90 kDa is also present in plasma membrane (lane 1)and cell lysate preparations (lane 2 and 4) from infected cells immunoprecipitated with anti-94 kDa IgG and anti-62 kDa IgG. No cross reacting proteins of similar M.W. were detected in plasma membranes from uninfected cells, with any of the above antisera (data not

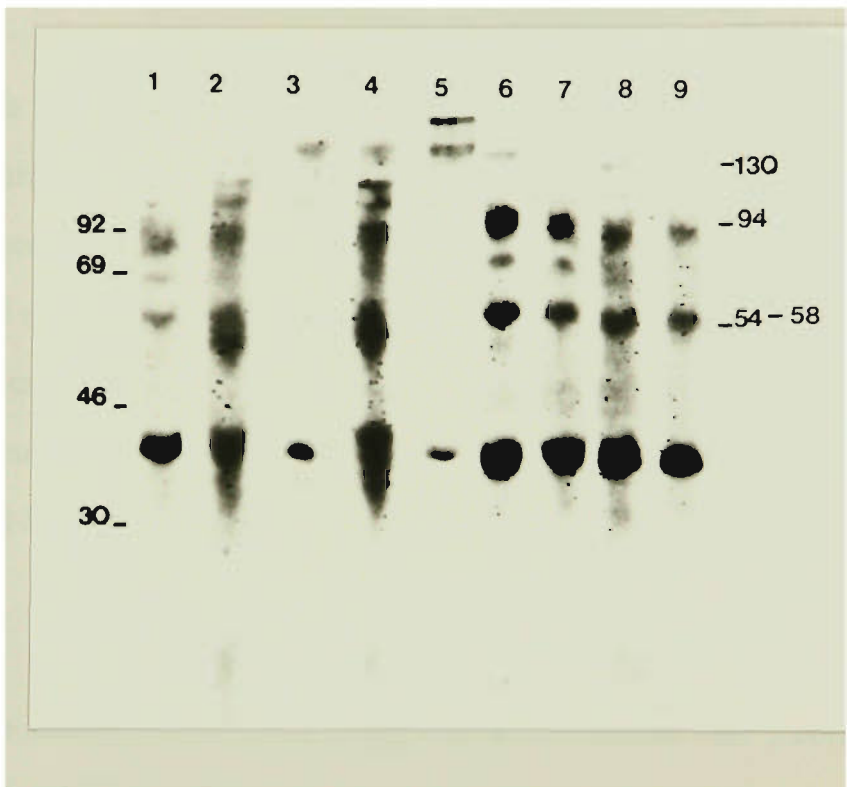


Figure 30. Autoradiograph of 10% SDS PAGE of infected and uninfected cell extracts and plasma membranes immunoprecipitated with antibodies. Plasma membranes from infected cells (lane 1), and cell lysates from infected and uninfected cells (lanes 2 and 3 respectively) were reacted with anti - 94 kDa IgG. Proteins from infected and uninfected cell lysates (lanes 4 and 5 respectively), and plasma membrane proteins from infected cells (lane 6) were reacted with anti- 62 kDa IgG. Plasma membranes from infected cells (lane 7) were reacted with anti- 54 kDa IgG. Plasma membranes from infected cells were reacted with monoclonal antibodies 28-2 and 7-17 (lanes 8 and 9 respectively).

shown). Minor protein bands of M.W. 130, 94 and 54-58 kDa were, however, detected in uninfected cell lysates immunoprecipitated with anti-94 IgG kDa and anti-62 kDa IgG. A protein of 43 kDa was detected in all protein fractions. This is presumed to be the IgG F_c receptor.

The fact that no proteins were immunoprecipitated from the uninfected cell plasma membrane preparations supports the suggestion that there are novel HMWC which give rise to subunit proteins of 92-94 and 55 kDa. The detection of minor labelled protein bands of 130, 94 and 54-58 kDa in uninfected cell lysates with anti-62 kDa IgG and anti-94 kDa IgG concurs with the results obtained from immunoprecipitates analysed under non reducing conditions, where labelled HMWC's of lower affinity than the HMWC's of the infected cell lysates, contained reduced proteins of 92-94 and 55 kDa detected by silver staining.

In addition to the above immunoprecipitation analysis carried out with plasma membrane preparations isolated by two phase polymer systems only, plasma membranes extracted by the FMA procedure (Section 2.12) were used in similar analyses. A summary of the results from immunoprecipitations performed in duplicate with HCMV-induced proteins reactive with anti- 54 kDa, anti- 62 kDa and anti- 94 kDa antisera is given in Table XII. Proteins with molecular weights identical to those found in preparations for uninfected cells have not been tabulated.

Plasma membranes prepared by the FMA procedure and immunoprecipitated with anti-94 kDa and anti-54 kDa sera differed in M.W. to plasma membrane proteins isolated from the two phase polymer systems (Table XII). These differences in part reflect differences in the procedures used. Firstly antisera and not immunoaffinity purified IgG from the antisera was used, and secondly *S. aureus* and not Protein A sepharose was used to precipitate the immunocomplexes. FMA cross links proteins, and it has been reported that proteins of high molecular weights, > 100kDa are under represented in these plasma

Table XII.

Plasma Membrane Proteins from HCMV-Infected HEF, and Proteins Isolated from the Supernatant Culture Fluid of Infected HEF Reactive with Anti- 54 kDa, Anti- 62 kDa and Anti- 94 kDa Sera as Analysed by Immunoprecipitation, Reducing SDS PAGE and Fluorography.

Antisera	54 kDa	62 kDa	94 kDa
Plasma membrane protein from HCMV-infected HEF	42, 54, 70	ND	17, 24, 54, 62, 66-70,
	80		74, 80, 84, 92, 96
			98, 100
Proteins from the supernatants of HCMV-infected HEF	76, 66, 52	52, 66	80, 76, 66, 18

Metabolically labelled proteins from plasma membranes of infected cells extracted by the FMA procedure and infected cell supernatants immunoprecipitated with antisera raised against plasma membrane proteins of 54, 62 and 94 kDa and analysed by reducing SDS PAGE and autoradiography. Molecular weights of proteins (x 10^3) are listed above. ND :- not determined.

membrane prepararations (Mc Clure *et al.*, 1979). A broad range of proteins reacted with anti- 94 kDa sera including proteins of 54, 62 and 92 kDa. Proteins of 42, 52, 70, and 80 kDa were reactive with anti-54 kDa sera.

Proteins from the supernatant fluids of infected cells were also immunoprecipitaed with antisera raised against plasma membrane proteins from infected cells (Table XII). One protein from the infected cell supernatants that was immunoprecipitaed by all three antisera was of M.W. 66 kDa. Additional proteins of 52 and 76 kDa were reactive with the anti- 54 kDa sera and proteins of 18,76 and 80 kDa also reacted with the anti- 94 kDa antisera. These proteins could represent infected cell plasma membrane proteins that are sloughed off from the cells. The significance of these proteins is unknown , of interest though is the fact that the antisera recognize proteins in the supernatants of infected cells that are similar in molecular weights to some of the proteins identified as virus induced novel plasma membrane proteins.

3.4.1. **Immunoblot Analysis**

Immunoblot analysis was carried out in conjunction with immunoprecipitation and immunoaffinity chromatography, in order to determine the reactivity of plasma membranes with various antisera. Plasma membranes isolated from HCMV-infected and uninfected cells 72 hrs post infection by the FMA extraction procedure were fractionated on 10% or 12% reducing SDS gels (Section 2.5) and transferred to nitrocellulose to facilitate immunoblotting with antisera raised against plasma membrane proteins 54, 62 and 94 kDa (Section 2.9). Bovine serum albumin (BSA) or casein were used as non specific protein blocking agents. The bound antisera was detected by chromogen-activated horse radish peroxidase conjugated to goat anti-rabbit IgG.(Section 2.9). The results are given in Table XIII. Anti-54 kDa sera (1:100 dilution) reacted specifically with FMA extracted plasma membrane proteins of 54, 32-36 and 28 kDa, and anti- 62 kDa sera with a protein of 62-64 kDa only. The 94 kDa sera reacted with a broad range of

Table XIII.

Summary of Plasma Membrane Proteins from HCMV-infected HEF Reactive with Anti-54 kDa, Anti-62 kDa and Anti-94 kDa Sera as Detected by Immunoblot Analysis of Plasma Membranes Fractionated by Reducing SDS Page.

Antisera Against Plasma Membrane Protein	Blocking Agent	Membranes (Infected HEF)	Membrane (Uninfected HEF)
54 kDa	BSA (1:50/1:100)	54 32 - 36 28	88 46 44
62 kDa	Casein (1:100) (1:500)	 62 - 64 62 - 62	 - -
94 kDa	BSA (1:50)	 62 54 52 36 28	 -

Immunoblot analysis of plasma membranes isolated by FMA extraction, fractionated by reducing SDS PAGE and reacted with dilutions antisera raised against HCMV induced plasma membrane proteins of 54, 62 and 94 kDa . The molecular weights (X 10^3) of stained proteins and the non specific protein blocking agent are given above.

proteins including proteins of M.W. 52-54 and 62 kDa. Proteins of 36 and 28 kDa were also detected with this antisera.

3.4.2. Immunoaffinity Absorption Chromatography.

Immunoaffinity absorption chromatography columns prepared with anti-54 kDa IgG and anti-62 kDa IgG were used to isolate plasma membrane proteins specific for these antibodies from HCMV-infected HEF (Section 2.10). The protein eluates were analysed by reducing SDS PAGE. The anti-54 kDa IgG and anti-62 kDa IgG were shown to be specific for proteins of 54 and 62 kDa, respectively from infected plasma membranes. Uninfected plasma membrane proteins, even at twice the protein concentration used for infected plasma membrane proteins did not react with the anti-54 kDa IgG or anti-62 kDa IgG (Roig Farran, personal communication).

3.5. Reactivity of HCMV-Induced Plasma Membrane Proteins with Antisera Raised against HCMV Virion Envelope Proteins.

To further characterise the structural and functional associations between high molecular weight disulphide-linked protein complexes and plasma membrane proteins of 54, 62 and 94 kDa, and the relatedness of these proteins to proteins of similar molecular weights identified in HCMV virions, monospecific polyclonal antisera and monoclonal antisera raised against virion envelope protein constituents

were used in immunoprecipitation analyses of plasma membranes from infected cells (Section 2.5.2). All plasma membrane proteins were isolated by two-phase polymer extraction procedures at 72 h after infection (Section 2.3.1), and cell lysates also extracted at 72 h p.i. were prepared according to the procedures described in Section 2.4.2.

3.5.1. Monospecific Polyclonal Antisera 6PP and 4PP.

Data obtained from the reactivity of serum 6PP with plasma membranes from infected and uninfected HFF's as determined by immunoprecipitation and SDS PAGE under denaturing and non-denaturing conditions is summarised from 8 independent experiments (Tables XIV and XV). Sera 4PP and 6PP were raised in guinea pigs against HCMV envelope material eluted from an immunoaffinity column (Gonczol *et al.*, 1986) prepared with monoclonals against the protein complex designated gA by Pereira *et al* (1984). Guinea pig serum 6PP recognised high molecular weight protein complexes of 280, 220, 150 , 130 and 98 kDa in infected cell lysates, and HMWC's 280, 150, 130 and 98 kDa in plasma membrane preparations from infected cells as determined by immunoprecipitation and 5% non-reducing SDS PAGE analysis and fluorography (Sections 2.12 and 2.13) (Figure 31 ; Table XIV). Immunoprecipitates of solubilised plasma membranes from infected cells reactive with serum 6PP and analysed by non-reducing gel electrophoresis on 7.5% acrylamide gels gave rise to three discreet HMWC's at 150 ,140 and 130 kDa (data not shown). No radiolabelled proteins were detected in the plasma membrane samples from uninfected fibroblasts whereas a HMWC of 120-130 kDa was detected in uninfected cell lysates.

These findings are, in part, consistent for a previously reported high molecular weight complex of 130 -150 kDa from infected cell lysates and virions identified with the same antisera (Gonczol *et al.*, 1986). The only other high molecular weight protein complex previously isolated from infected cell lysates with serum 6PP was reported to be >200 kDa. The extended time of electrophoresis as well as the specific elution of immune complexes with 0.2M glycine pH 2,0 (Section 2.13) allowed for the isolation of several

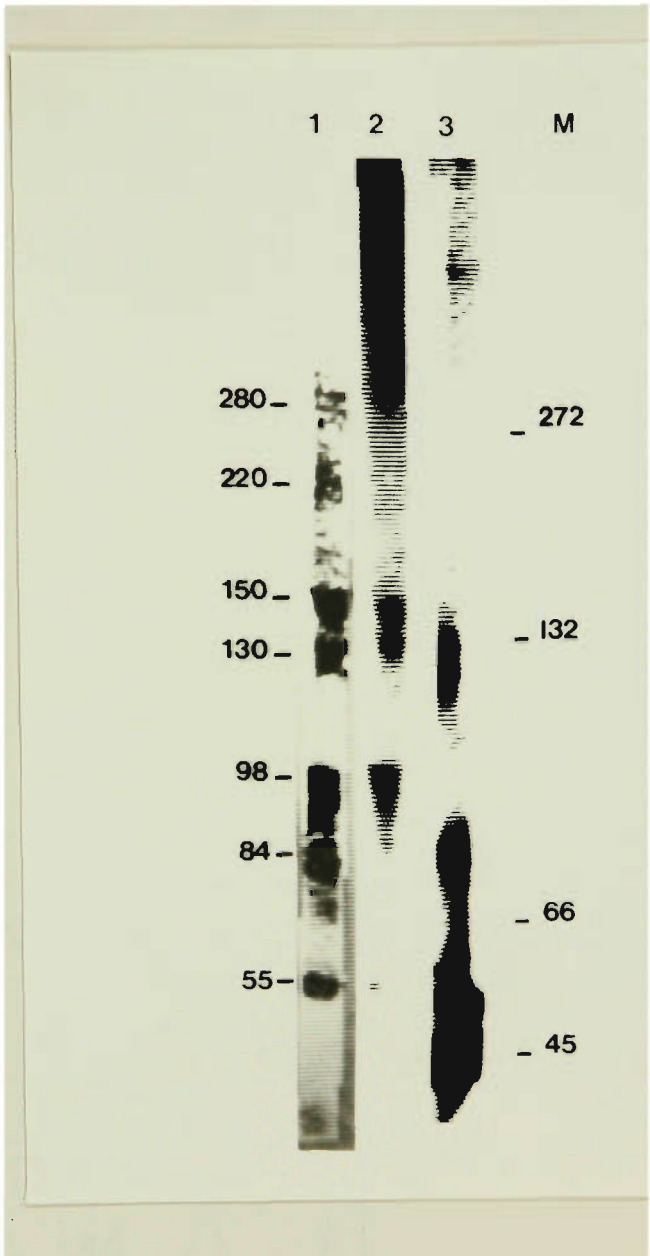


Figure 31 L^{35} [S] methionine labelled cell extracts and plasma membranes isolated 72 hrs post infection by two phase polymer extraction and immunoprecipitated with guinea pig serum 6PP.

Image enhanced autoradiograph of 5% non-reducing SDS PAGE of whole cell protein lysates from infected (lane 1) and uninfected (lane 3) fibroblasts, and plasma membranes (lane 2) isolated from infected fibroblasts. No proteins were detected in immunoprecipitates from plasma membranes isolated from uninfected cells (not shown). Molecular weights ($X10^3$) of disulphide linked high molecular weight protein complexes and markers (M) are given.

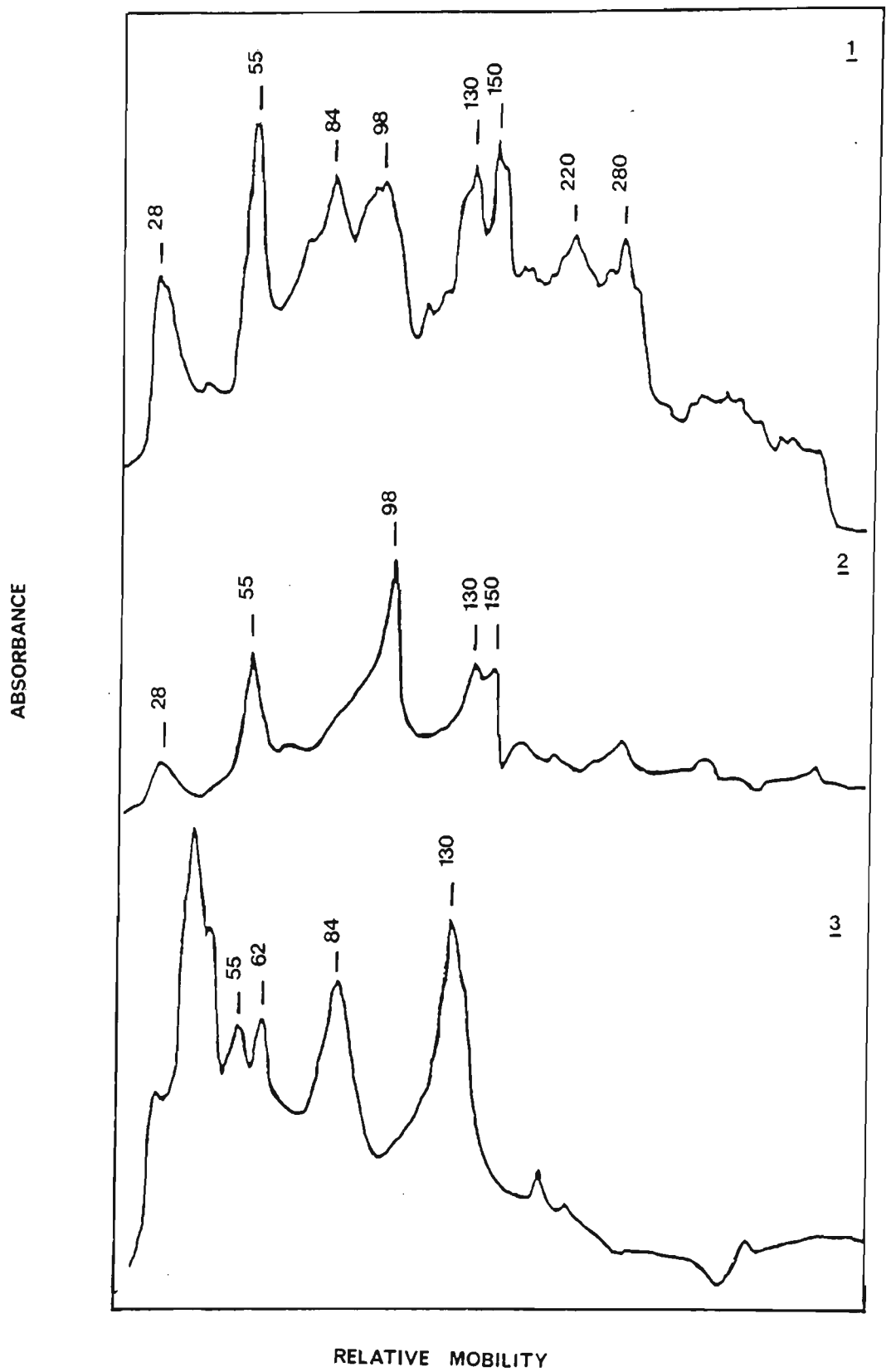


Figure 31b. Laser densitometer scans of protein complexes reported in Figure 31.

discrete HMWC listed above in both the cell lysates and purified plasma membranes from infected cells. Since silver staining of some the non reducing gels indicated the presence of immunoprecipitated HMWC's in the uninfected cell preparations, unlabelled HMWC from plasma membrane proteins from uninfected cells, (data not shown) were excised from the gel in the same relative positions as labelled complexes from plasma membrane proteins of infected cells. Results obtained from the reduction of HMWC's with either 2% or 4% 2-ME and subsequent analysis of the HMW protein complexes by re-electrophoresis and silver staining to establish the constituent sub-unit proteins are represented in Figures 28, 32 and 33 and Table XV.

The sub-unit composition of HMWC's of 120-130 and 80-85 kDa in the uninfected cell lysates that cross-reacted with serum 6PP were not examined. These molecular weights differed significantly from those found in infected cell lysates. All the complexes from both the plasma membranes and the cell lysate extracts contain a protein of 54-58 kDa. A protein of 26-28 kDa was also evident after reduction of HMWC 150 kDa from both the plasma membrane extracts (Figure 28) and whole cell lysates of infected cells (Figure 32), and for the HMWC 280 kDa from the plasma membranes. Furthermore two proteins were detected in the 54-58 kDa molecular weight range when the latter HMWC was reduced (Figure 32, lane 1). A protein of 26K-28K has not been previously reported as a sub-unit component of the 130 -150 kDa HMWC recognised by this antisera. The additional treatment of HMWC of 150 kDa from plasma membranes from infected and uninfected cells with 4% 2 ME indicated reduced proteins of 55 and 28kDa in the infected sample only.

However, unlabelled HMWC's of 150 and 140 kDa from plasma membranes of uninfected cells contained subunit proteins in the region of 94 and 55 kDa, although the HMWC 140 kDa in plasma membranes from uninfected cells appears to be slightly <55 kDa and there is no protein at 28 kDa (Figure 33). The sub unit proteins contained in the HMWC located in the plasma membranes of uninfected cells were in substantially smaller

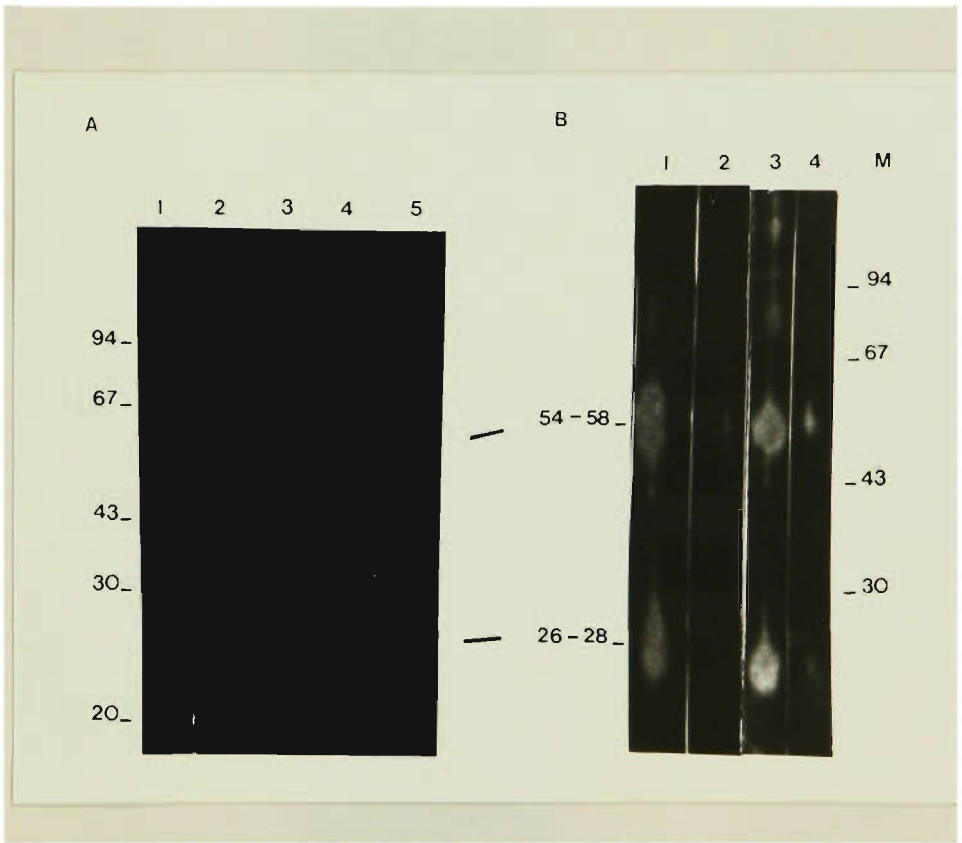


Figure 32. Re-electrophoresis on 10% reducing SDS PAGE of disulphide linked protein complexes isolated under non-reducing conditions from infected cell extracts (A) and plasma membranes (B) by immunoprecipitation with serum 6PP (refer Fig. 31). Reduction of HMWC's of the following molecular weights ($X10^3$) are given: 280 kDa (A, lane 2), 220 kDa (A, lane 3), 150 kDa (A, lane 4), 98 kDa (A, lane 5), 280 kDa (B, lane 1), 98 kDa (B, lane 2), 150 kDa (B, lane 3) and 130 kDa (B, lane 4). Molecular weight markers ($X10^3$) are indicated (A, lane 1).

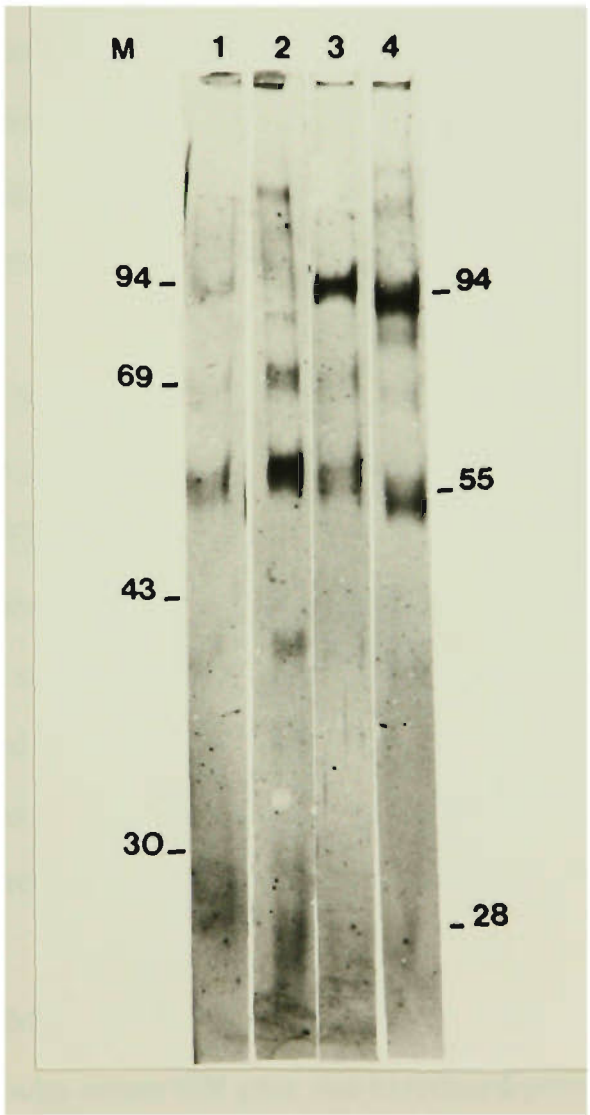


Figure 33. Silver stain of 10% reducing SDS PAGE analysis of HMWC 150 kDa of plasma membrane preparations immunoprecipitated with serum 6PP from infected and uninfected cells (lanes 1 and 3 respectively) and HMWC 140 kDa of plasma membranes immunoprecipitated with the same antisera from infected and uninfected cells (lanes 2 and 4 respectively). Molecular weights ($X10^3$) of proteins and markers (M) are indicated.

quantities than those of similar molecular weights contained in the HMWC from infected cells.

Guinea pig serum 4PP immunoprecipitated a HMWC of 130-150 kDa from plasma membrane preparations from infected cells and a HMWC of 250 kDa from plasma membrane preparations from uninfected cells as determined by non-reducing 7.5%. SDS PAGE and fluorography (Figure 34 ; Table XIV). No HMWC of 130-150 kDa was seen in the uninfected cells.

Reduction of the HMWC 130-150 kDa with 2ME, re-electrophoresis and silver staining demonstrated the presence of a major protein of 54 -58 kDa and minor proteins of 130, 94 and 28 kDa (Figure 35 ; Table XVI). This indicates that the HMWC's of the plasma membrane proteins from infected cells recognised by serum 4PP and 6PP, are essentially similar if not the same. Serum 4PP recognised labelled plasma membrane glycoproteins from the infected cell of M.W. 250 , 130 , 86 , 66 and 52 kDa under reducing SDS PAGE conditions (Figure 36). Proteins of similar M.W. have been reported for virion envelope glycoproteins immunoprecipitated with 4PP (Gonczol, *et.al.*, 1986).

The labelled HMWC of 250 kDa immunoprecipitated from plasma membranes of uninfected cells with serum 4PP gave rise to reduced proteins, detected by silver staining of similar molecular weights to that of the HMWC of 130-150 immunoprecipitated from plasma membranes from infected cells (Figure 39 ; Table XVI). However, reduction and re-electrophoresis of the unlabelled HMWC of 130-150 immunoprecipitated from uninfected cells in the presence of 4% 2ME gave rise to a single protein band at approximately 55 kDa (Figure 40). As such the HMWC of 130-150 kDa immunoprecipitated from infected cell plasma membranes with serum 4PP appears to be unique to virus infected cells.

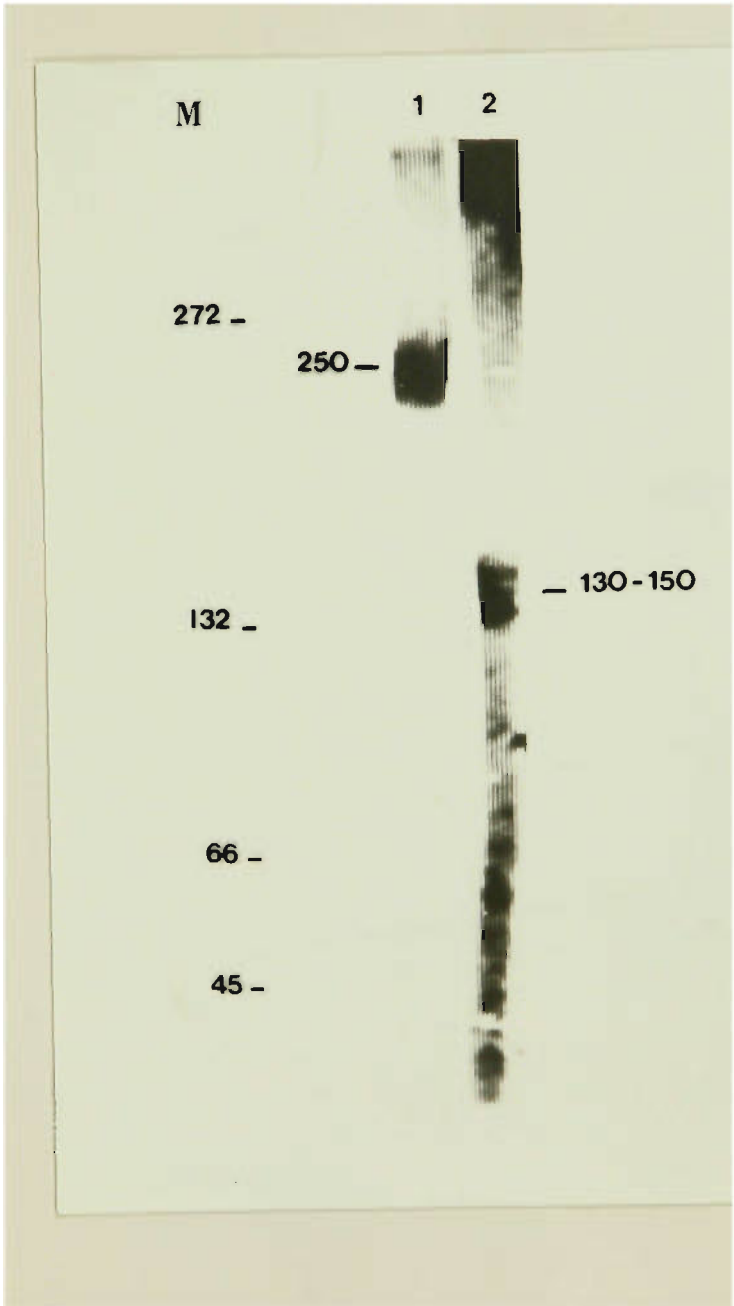


Figure 34. L^{35} [S] methionine labelled membrane glycoproteins isolated 72 hrs post infection by two phase polymer extraction and immunoprecipitated with guinea pig serum 4PP.

Image enhanced autoradiograph of 7.5% non-reducing SDS PAGE analysis of plasma membranes isolated from uninfected fibroblasts (lane 1) and HCMV infected fibroblasts 72 hrs post infection (lane 2). Molecular weights ($X10^3$) of high molecular weight protein complexes and non-reduced markers (M) are indicated.

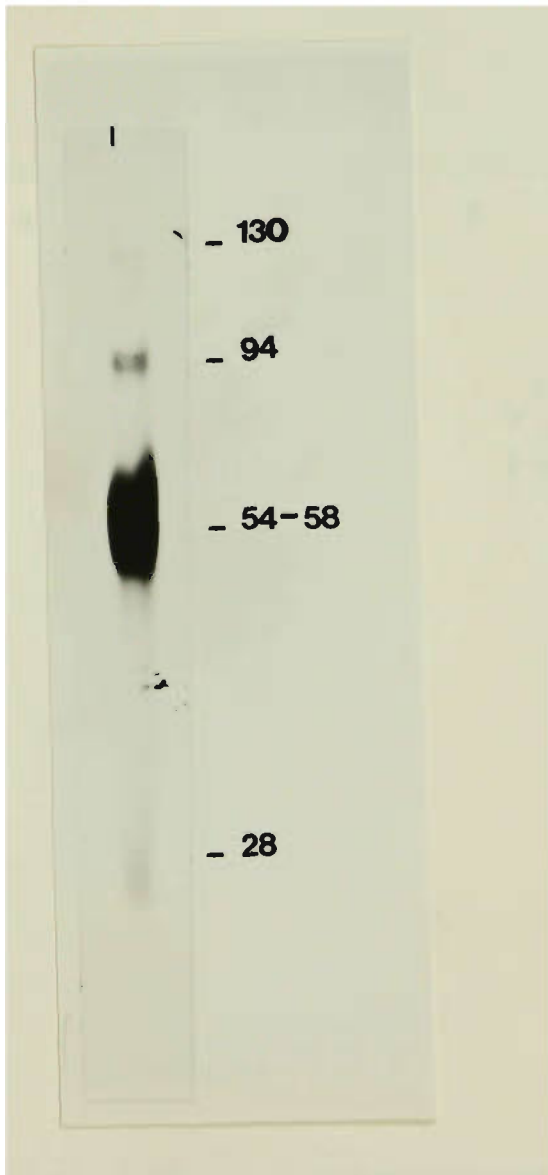


Figure 35. Re-electrophoresis of disulphide linked protein complexes isolated from plasma membranes from infected fibroblasts by immunoprecipitation with serum 4PP and gel analysis under non-reducing conditions (refer Fig. 4, lane 2). Silver stain of 7.5%-15% gradient SDS PAGE analysis of HMWC of 130-150 kDa (lane 1). Molecular weights ($X10^3$) of reduced protein components are given.

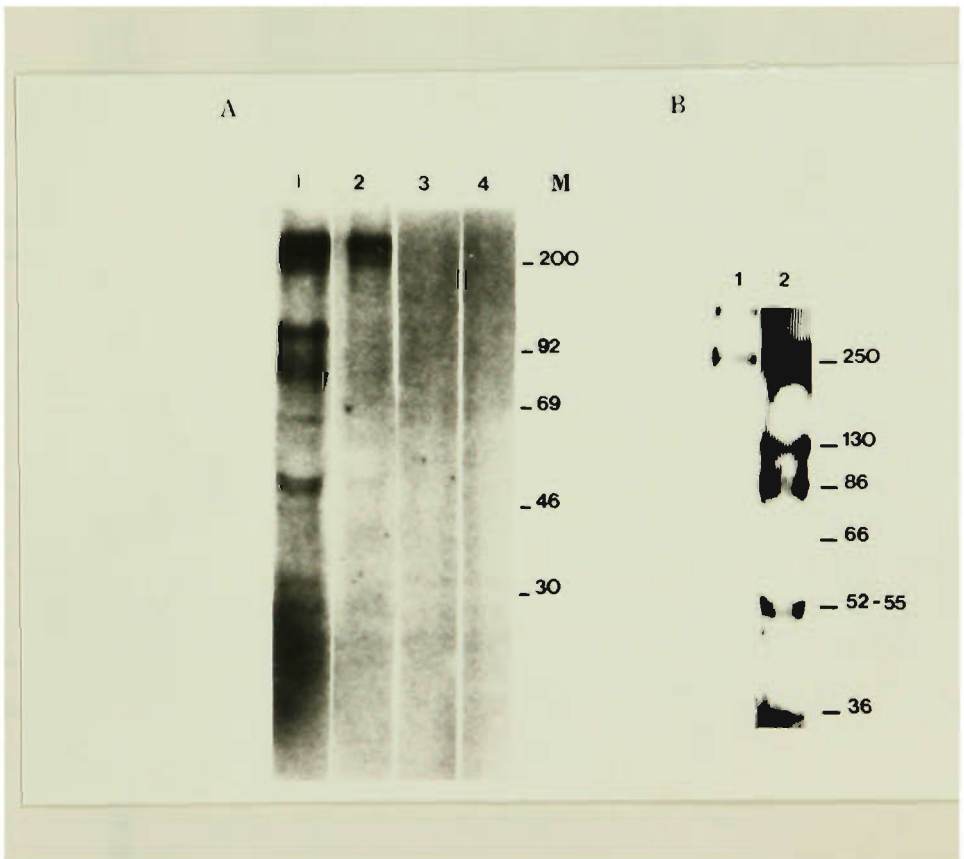


Figure 36a. SDS PAGE analysis of L^{35} [S] methionine labelled membrane proteins isolated 72 hrs post infection by two phase polymer extraction and immunoprecipitation. Autoradiograph of 10%-20% gradient SDS PAGE analysis of plasma membrane proteins from infected and uninfected fibroblasts immunoprecipitated with either serum 4PP (lanes 1 and 2) or pre-immune guinea pig serum (lanes 3 and 4).

Figure 36b. Image enhanced autoradiograph of 10%-20% gradient reducing SDS PAGE analysis of L^{35} [S] methionine labelled plasma membrane glycoproteins isolated from uninfected fibroblasts (lane 1) and HCMV infected fibroblasts (lane 2) immunoprecipitated with serum 4PP. Molecular weights of proteins are indicated.

Table XIV.
HMWC's and Reduced Proteins of HCMV Infected and Uninfected Plasma Membranes and Cell Lysates Immunoprecipitated by Guinea Pig Serum.

	Infected		Uninfected	
	m	l	m	l
Serum 6PP				
HMWC				
72h p.i.	-	280	-	-
	-	220	-	-
	150	150	-	-
	130	130	-	120
	94-98	98	-	-
	-	84	-	80
	55	55	-	-
	28	28	-	-

	Infected	Uninfected
	m	m
Serum 4PP		
HMWC's		
72 h. p.i	130-150	250
48 h. p.i.	150	
	130	
	94-98	
	55	
12 h. p.i.	130-150	
	94-98	
Reduced Proteins.		
72 h. p.i.	250	250
	130	
	86	
	66	
	52-55	
	36	
48 h.p.i.	150	
	130	
	94	
	74-68	
	52-54	
12 h. p.i.	150	
	130	
	94	
	74	
	68	
	52	

HMWC's of HCMV infected and uninfected cell plasma membranes and cell lysates immunoprecipiated with serum 6PP and 4PP and analysed by non reducing SDS PAGE and autoradiography. Immunoprecipitated proteins reactive with srum 4PP from infected and uninfected cell plama membranes were also analysed directly by reducing SDS PAGE. Molecular weights (x 10^3) of proteins are listed above.

In summary it can be seen that serum 4PP contained antibodies of higher affinity and greater specificity for the HMWC 130-150 kDa than did serum 6PP. This is partially explained by the fact that serum 4PP was raised against the first set of proteins eluted from an immunoaffinity column prepared with a Mab reactive with a set of infected cell proteins now termed the gB complex of glycoproteins. Serum 6PP recognized several more complexes, and appears to have a lower affinity for the 130-150 kDa protein and to react with addtional epitopes. Under reducing conditions both sera reacted with protein constituents of the gB protein complex.

As several high molecular weight protein complexes from plasma membrane and cell protein fractions from uninfected cells were recognized by sera specific for virus proteins, it was decided to investigate whether the expression of any of these proteins varied during the course of infection. These experiments were carried out primarily to determine whether or not proteins reactive with sera raised against virion envelope proteins are expressed at earlier times post infection in the plasma membranes of infected cells.

3.6. Time Course Immunoprecipiation Analysis of Plasma Membrane Proteins Reactive with Serum 4PP.

It was considered important to establish when after infection, proteins reactive with serum 4PP, appeared in the plasma membranes. All previous analyses undertaken had been at 72h p.i. , hence, three sets of time course immunoprecipitation experiments with cells labelled at at 12 h and 48 h p.i. were carried out with serum 4PP. The plasma membrane proteins extracted from infected cells at 12h and 48h p.i. reactive with serum 4PP were analysed by non reducing and reducing SDS PAGE and autoradiography. Labelled and unlabelled HMWC's were also excised from the gels, reduced with 2% and/or 4% 2 ME and re-electrophoresed. The data represented here is a summary of those results.

Labelled non reduced high molecular weight protein complexes of 150, 140, 130 and 94 kDa from plasma membranes, isolated from infected cells at 12 h and 48 h p.i. were recognised by serum 4PP (Figure 37). A HMWC of 250 kDa (data not shown) and a protein of 55 kDa was also isolated at 48 h p.i. The HMWC of >250 kDa and proteins at 94 and 55 kDa were previously not detected in the plasma membrane sample isolated at 72 h p.i. (see Figure 34). The amount of radiolabelled protein immunoprecipitated with serum 4PP from plasma membranes isolated at 48 h p.i. was greater than that at 12 h p.i.

Immunoprecipitation analysis of plasma membranes isolated from infected cells at 12 h and 48 h p.i., with serum 4PP analysed under reducing SDS PAGE conditions demonstrated proteins of M.W. 150, 130, 94, 68, and 52-54 kDa. As with the proteins analysed under non-reducing SDS PAGE, the amount of protein isolated at 48 h p.i. was much higher than that at 12 h p.i. This fact is clearly evident when the HMWC were re-electrophoresed (Figure 40) ; HMWC protein constituents from the 12 h isolation were indetectable with siver staining.

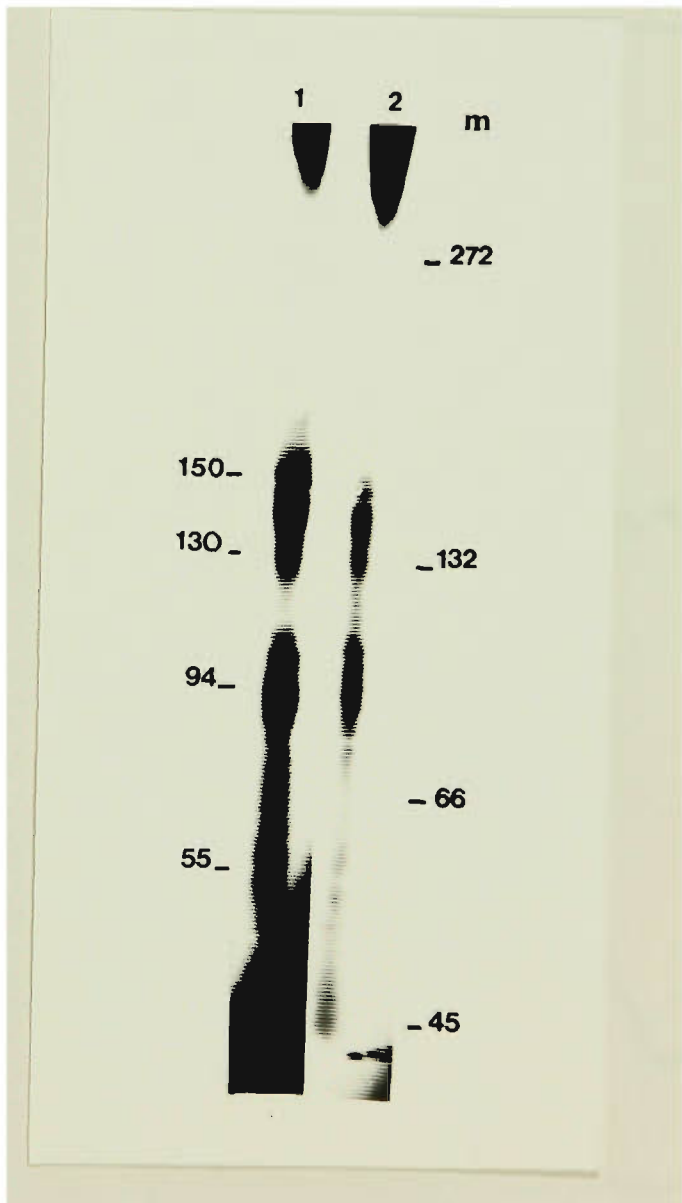


Figure 37. Time course analysis of L^{35} [S] labelled plasma membrane proteins isolated from infected fibroblasts at 12 hrs and 48 hrs post infection by two phase polymer extraction and immunoprecipitated with serum 4PP.

Autoradiograph of 7.5% non-reducing SDS PAGE analysis of plasma membranes isolated at 48 hrs (lane 1) and 12 hrs (lane 2) post infection. Molecular weights ($X10^3$) of protein complexes and non-reduced markers (M) are indicated.

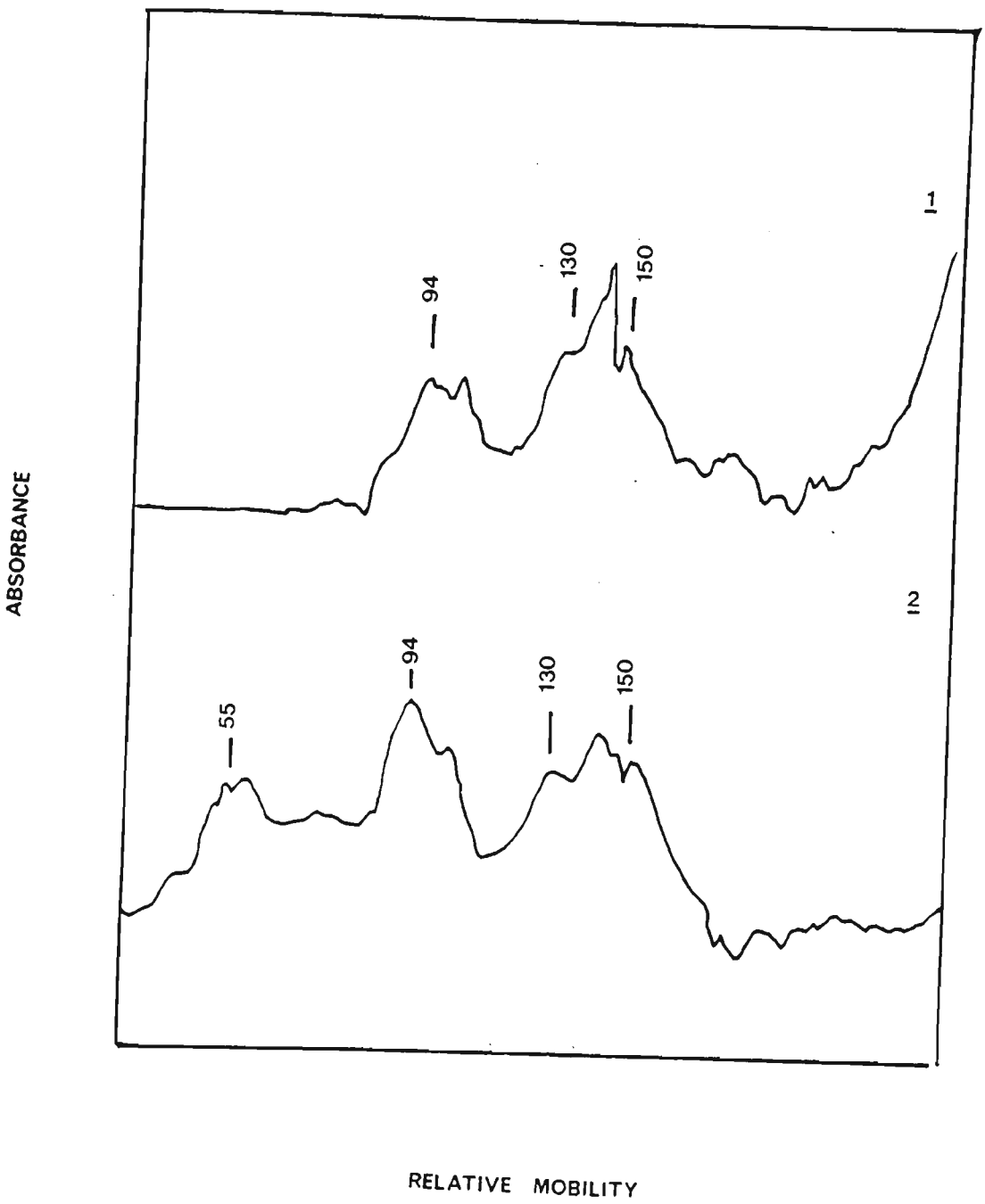


Figure 37b. Laser densitometer scan of HMWC's of plasma membrane proteins at 12 hrs (1) and 48 hrs (2). Molecular weights ($X10^3$) are indicated.

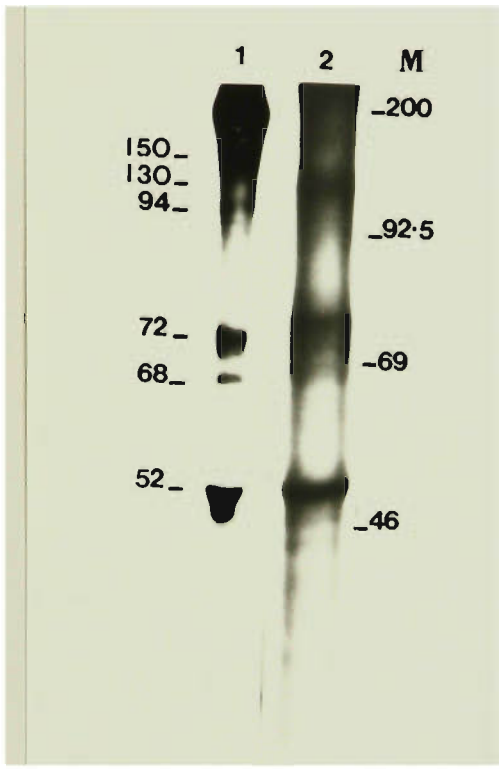


Figure 38a. Time course analysis of L^{35} [S] labelled plasma membrane proteins isolated from infected fibroblasts at 12h or 48h post infection by two phase polymer extraction and immunoprecipitated with serum 4PP.

Autoradiograph of gradient 10%-20% SDS PAGE analysis of plasma membrane proteins isolated at 12h (lane 1) and 48h (lane 2) post infection. Molecular weights ($X10^3$) of proteins and markers (M) are given.

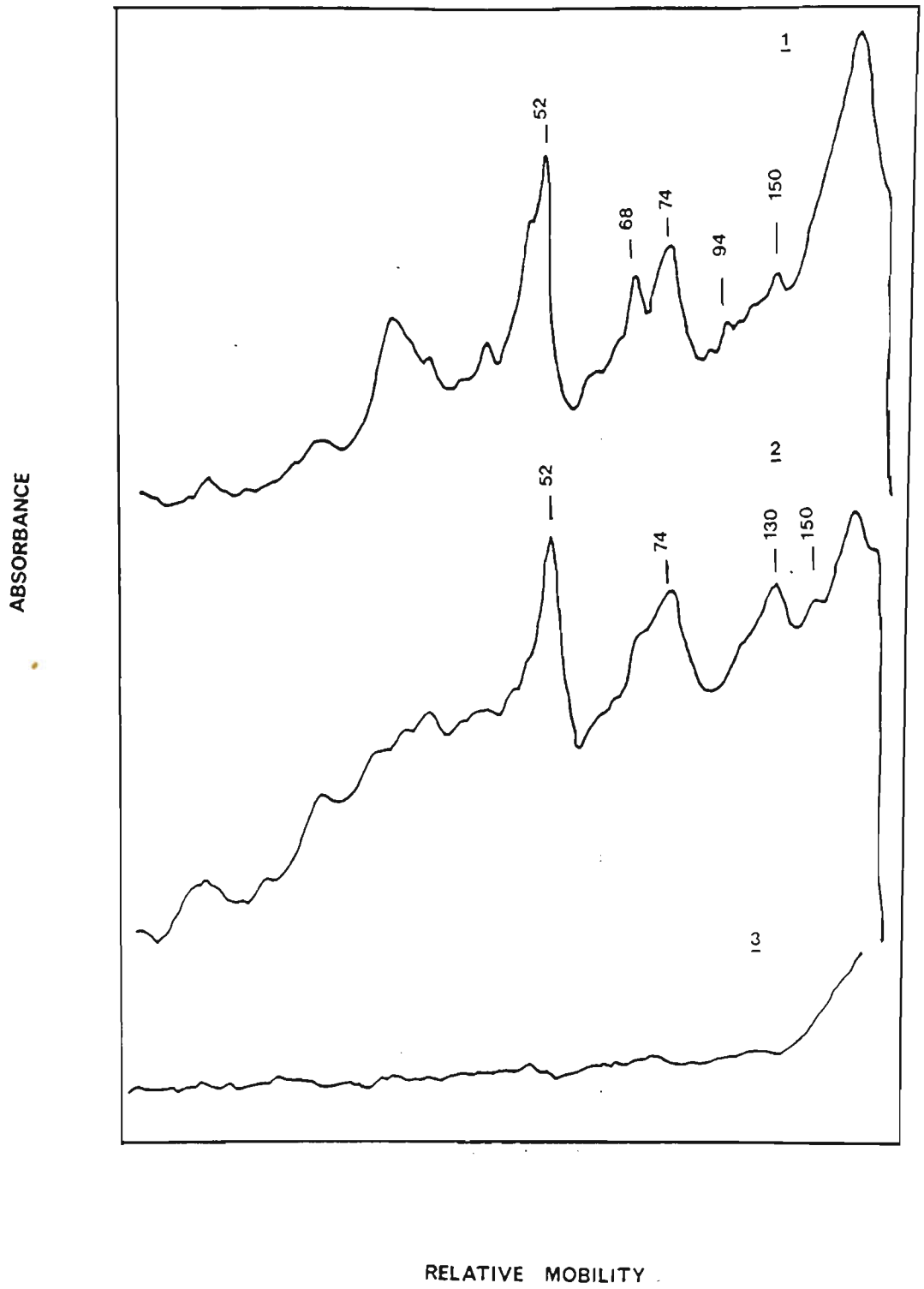


Figure 38b. Laser densitometer scan of plasma membrane proteins isolated at 12 hrs (1) and 48 hrs (2), analysed by reducing SDS PAGE. Molecular weights ($X10^3$) of protein peaks are indicated.

Table XV.

Reduction and Re-electrophoresis of HMWC's from Plasma Membranes and Cell Lysates From HCMV Infected and Uninfected Cells Immunoprecipitated with Serum 6PP.

Membrane	Infected		Unifected	
	m	l	m	l
HMWC's				
>500	160	ND	-	ND
	150		150	
	-		130	
	92		92	
	68		-	
	55		55	
	28		-	
280	54-55	54-55	-	-
	26-28	-	-	-
220	ND	54-55	ND	-
		26-28		-
150	130	-	-	-
	94	-	94	-
	68-70	-	-	-
	54-58	54-58	55	-
	26-28	26-28	-	-
140	130	-	-	ND
	-	-	94	
	68	-	-	
	55	-	55	
130	54-58	-	-	
98	54-58	54-55	-	

Reduction and re-electrophoresis of HMWC's from plasma membranes and cell lysates of HCMV infected and uninfected cells immunoprecipitated with serum 6PP and analysed by reducing SDS PAGE and silver staining. Molecular weights (x 10^3) of proteins are listed above.

Table XVI.

Reduction and Re-electrophoresis of HMWC's from Plasma Membranes and Cell Lysates from HCMV Infected and Uninfected Cells Immunoprecipitated with Serum 4PP.

4PP	Infected	Uninfected
	m	m
72h. p.i.		
250	ND	130
		94
		86
		55
130-150	130	-
	94	-
	54-58	55
	28	-
48h. p.i.		
250	160	
	150	
	130	
	94	
	86	
	55	
130-150	130	
	94	
	86	
	55	
12h. p.i.		
250	94	
130-150	130	
	94	
	86	
	55	

Reduction and re-electrophoresis of HMWC's from plasma membranes of HCMV infected and uninfected cells immunoprecipitated with serum 4PP and analysed by reducing SDS PAGE and silver staining. Molecular weights (x 10^3) of proteins are listed above

Reduction with 2% 2ME and re-electrophoresis of the HMWC 250 kDa isolated from plasma membranes from infected cells at 48 h. p.i. yielded two major protein bands of 94 and 58 kDa and minor protein bands of 150, 130 and 86 kDa as detected by silver staining (Figure 39). Reduction of the HMWC of 250 kDa from both plasma membranes isolated at 12 h p.i. and plasma membranes from uninfected cells yielded a protein of 94 kDa. In contrast, reduction and re-electrophoresis of the 130-150 kDa protein isolated at 12 h and 48 h p.i. from infected cell plasma membranes yielded proteins of 94, 86 and 55 kDa (Figure 39). No reduced proteins were detected in the 130 -150 kDa protein isolated from plasma membranes from uninfected cells .

Since reduction with 2% 2ME was considered to be partially incomplete for some of the HMWC's (primarily due to the amount of protein in some of the HMWC's) some of these complexes were re-treated with 4% 2ME to ensure reduction of all disulphide bonds. It should be noted that partial reduction could also be due to the folding of the protein rendering some disulphide bonds inassessible to the action of the 2 ME.

When the HMWC's of 250 and 130-150 kDa from plasma membrane proteins isolated from uninfected cells and those isolated at 12h, 48h and 72h p.i. were reduced with 4% 2ME differences in the protein profiles were observed between the infected cell proteins isolated at 72h p.i. and those from the complexes from uninfected cells and the 12h and 48h sample (Figure 40). The 140 and 150 kDa complexes for the 72h p.i. sample reduced to constituent proteins of 94 and 28 kDa as well as what appears to be two proteins in the 54-54 kDa range. The complexes from the other samples reduced to only one protein in the 54-58 kDa range. Further the presence of two proteins in the 54-58 kDa region, the lower protein probably corresponding to the 54 kDa proteins detected in the reduced HMWC's of 150,140 and 130 kDa of plasma membranes isolated at 48h p.i. and immunoprecipitated with serum 4PP (Figure 40, lanes 1, 2 and 3)). This could indicate that the upper 54-58 kDa protein of the reduced HMWC's of 150 and 140 kDa in the plasma membranes isolated at 72h p.i.(Figure 40, lanes 4 and 5) represents a host cell

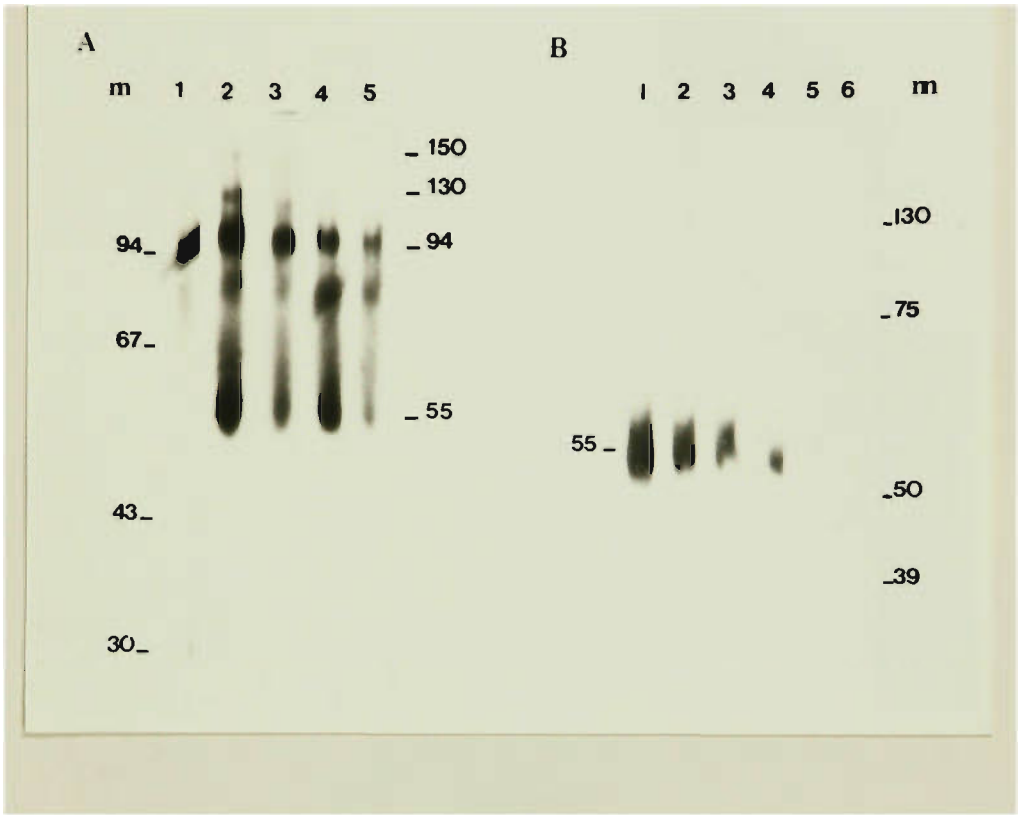


Figure 39a

and 39b. Re-electrophoresis of HMWC's obtained from immunoprecipitation of plasma membranes from HCMV infected and uninfected cells with serum 4PP.

Re-electrophoresis in the presence of 2% 2ME of HMWC of 250 kDa from plasma membranes isolated at 12h. and 48h. p.i. (A: lanes 1 and 2 respectively). HMWC of 130 - 150 kDa also from plasma membranes isolated at 12h and 48 h. p.i. were similarly treated (A: lanes 4 and 5 respectively). Lane A 3 represents the re-electrophoresis in 2% 2ME of HMWC 250 kDa of plasma membranes isolated from uninfected cells. Re - electrophoresis of HMWC's 250 and 130-150 kDa in the presence of 4% 2ME (Fig. 39b). HMWC of 250 kDa of plasma membranes from uninfected (B lane 1) and infected cells isolated at 48h p.i (B lane 4) and 12h p.i. (B lane 6). HMWC of 130-150 kDa of plasma membranes from uninfected cell (B lane 2) and infected cells isolated at 48 h p.i.(B lane 3) and 12 h p.i.(B lane 5).

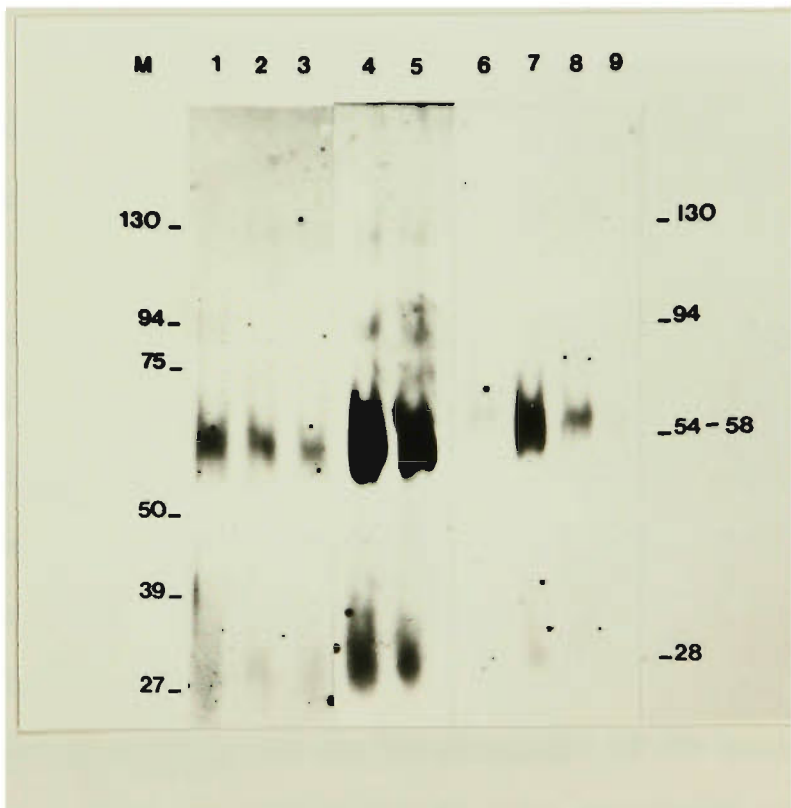


Figure 40. Re-electrophoresis on 7.5%-15% gradient SDS PAGE of disulphide linked protein complexes isolated under non-reducing conditions from plasma membranes from infected fibroblasts at 12 hrs or 48 hrs post infection by immunoprecipitation with serum 4PP.

Silver stain of reduced HMWC's of 150 kDa (lane 1), 140 kDa (lane 2), and 130 kDa (lane 3) from plasma membranes isolated at 48 hrs post infection; HMWC's of 150 kDa (lane 4), 140 kDa (lane 5) and 130kDa (lane 6) from plasma membranes isolated at 72 hrs post infection and HMWC's of 150 kDa (lane 7), 140 kDa (lane 8) and 130 kDa (lane 9) isolated from membranes from uninfected fibroblasts.

protein as indicated by the position of the 54-58 kDa proteins detected for reduced HMWC of 150 and 140 kDa from plasma membranes of uninfected cells..This suggests that serum 4PP only detects the unique virion coded complex at 72h p.i., having subunit components of 94, 54-58 and 28 kDa. The presence of a 62 kDa protein in this complex cannot be clearly ascertained due to the intensity of the bands in the 54-58 kDa region.

3.7. Reactivity of HCMV-Induced Plasma Membrane Proteins with Monoclonal Antibodies Specific for the Virion Envelope Glycoprotein Complex gB.

Since proteins from plasma membranes and cell lysates of uninfected fibroblasts cross reacted with antisera raised against HCMV induced plasma membrane proteins and virion envelopes, monoclonal antibodies specific for the virion gB glycoprotein complex were used in similar analysis to examine whether or not this phenomenon was due to non specific antisera or whether plasma membrane and cell proteins from uninfected cells contained epitopes similar to the gB proteins. The purposes of using the monoclonal antibodies was also to examine whether the production of the virion gB proteins substantially increases during infection and whether these proteins are incorporated into the plasma membrane.

Plasma membranes and cell lysates isolated from HCMV infected cells at 12 h, 48 h and 72 h after infection were immunoprecipitated with Mab 15D8 and analysed by non-reducing and reducing SDS PAGE and fluorography (Sections 2.5, 2.12 and 2.13).

Non-reducing gel analysis demonstrated the occurrence of high molecular weight protein complexes of 130-150 and 250 kDa in plasma membranes isolated at 72 h p.i., and a HMWC 130 -150 kDa in cell lysates from infected cells. However, HMWC's of 250 k Da were also immunoprecipitated from plasma membrane and cell lysate preparations from uninfected cells, although no other HMWC's could be detected for this time period

of exposure of the autoradiograph. Reduction and re-electrophoresis of the plasma membrane 130-150 kDa complex from infected cell plasma membranes, followed by fluorography shows that the HMWC 130-150 kDa is comprised primarily of sub-unit proteins of 92 and 55 kDa (Figure 42). Three minor protein bands of 150, 130 and 68 kDa were also present. The 150 and 130 kDa may represent residual HMWC proteins resulting from incomplete reduction of disulphide-linked monomers. Reduction and re-electrophoresis of the unlabelled HMWC of 130-150 kDa from uninfected cell plasma membranes resulted in a protein of 55 kDa as detected by silver staining (data not shown) and is the same as the results of the uninfected cell complexes obtained with serum 4PP and anti-54 kDa IgG.

An analysis of proteins reactive with Mab 15D8 isolated under reducing conditions from plasma membranes and whole cell lysates isolated at 72 h p.i. demonstrated proteins of M.W. 150, 130, 92-94, 68, 55 and 25 kDa (Figure 43 : Table XVII). No proteins were detected for plasma membrane and cell lysate preparations from uninfected cells or infected cells at 12h and 48h p. i. when the autoradiographs were exposed for three days, except for a high molecular weight protein of ~ > 200 kDa, detected in all in aformentioned samples (Figure 43). Proteins of > 200, 130, 92-94 and 55 are similar in molecular weights to the set of proteins isolated from infected cell lysates and virions with Mab 15D8 (Rasmussen *et al.*, 1985a). Minor differences in the proteins profiles obtained under reducing conditions for whole cell lysate proteins and purified plasma membrane proteins are present. The amount of the 55 and 28 kDa proteins obtained for the cell lysate fraction is negligible, compared to that of the plasma membrane fraction.

At 3 days exposure of the autoradiograph, only plasma membrane proteins isolated at 72 h p.i. could be detected (Figure 41 and 43). Long-term exposure of both the reducing and non-reducing gels to the x-ray film (2 weeks) revealed the presence of plasma membrane and cell lysate proteins immunoprecipitated with Mab 15D8 at 12 h and 48 h

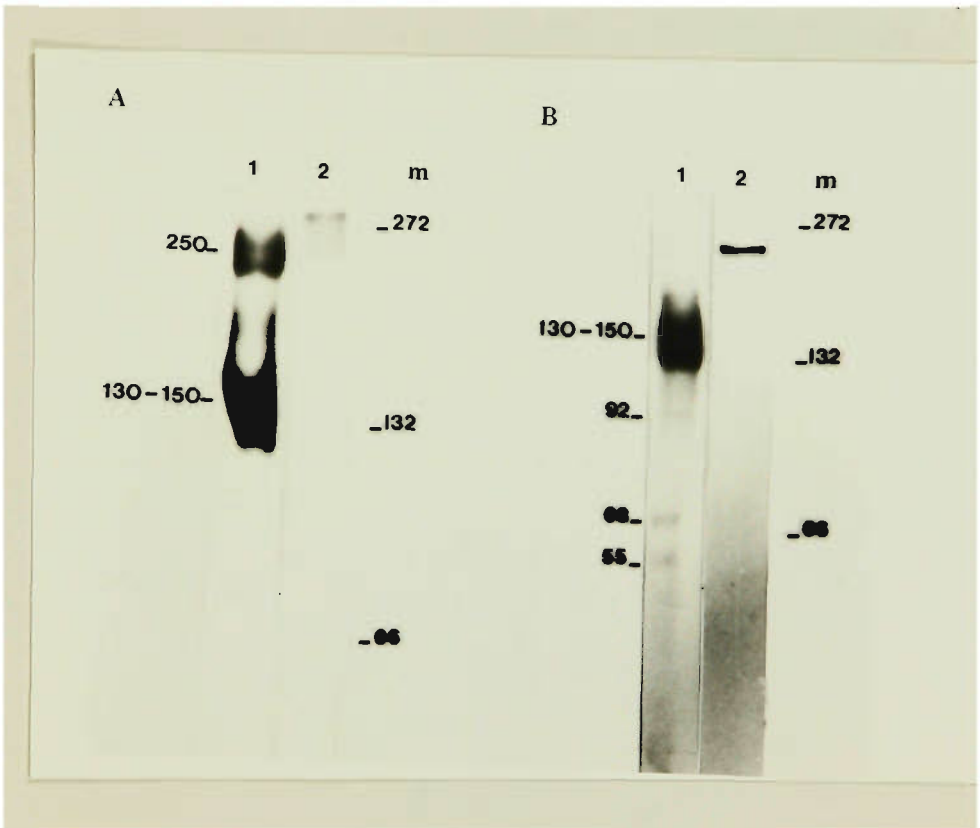


Figure 41. Immunoprecipitates of L^{35} [S] methionine labelled proteins with Mab 15D8. Autoradiograph of 7.5% non-reducing SDS PAGE analysis of plasma membranes from HCMV infected (A lane 1) and uninfected (lane 2) fibroblasts, B. cell lysates, extracted with 1% NP-40 and 1% sodium deoxycholate from HCMV infected (lane 1) and uninfected (lane 2) fibroblasts. Molecular weights ($X10^3$) of disulphide linked protein complexes and non-reduced markers (M) are indicated.

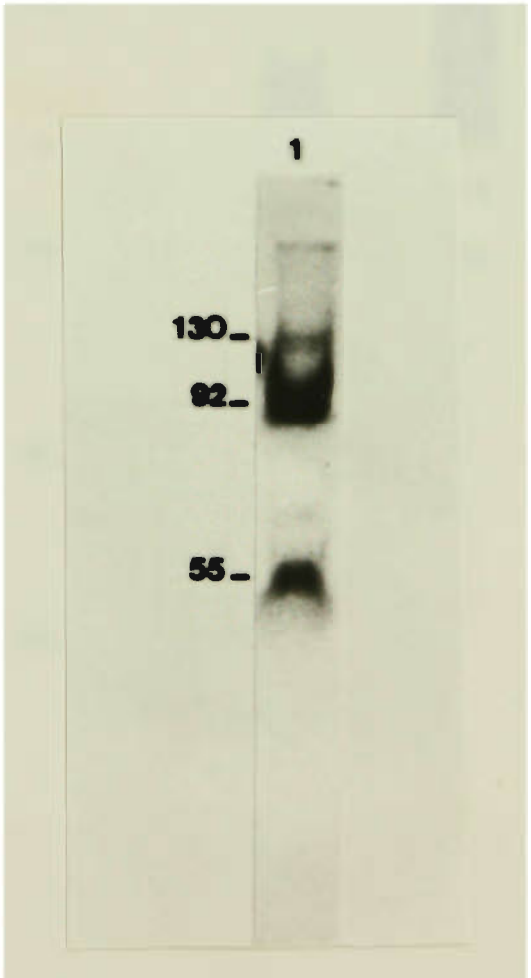


Figure 42. Autoradiograph of 10% SDS PAGE of HMWC 130-150 kDa immunoprecipitated with Mab 15D8 (Fig. 1A, lane 1) re-electrophoresed in the presence of 4% 2-ME. Molecular weights of plasma membrane proteins comprising the HMWC are indicated.

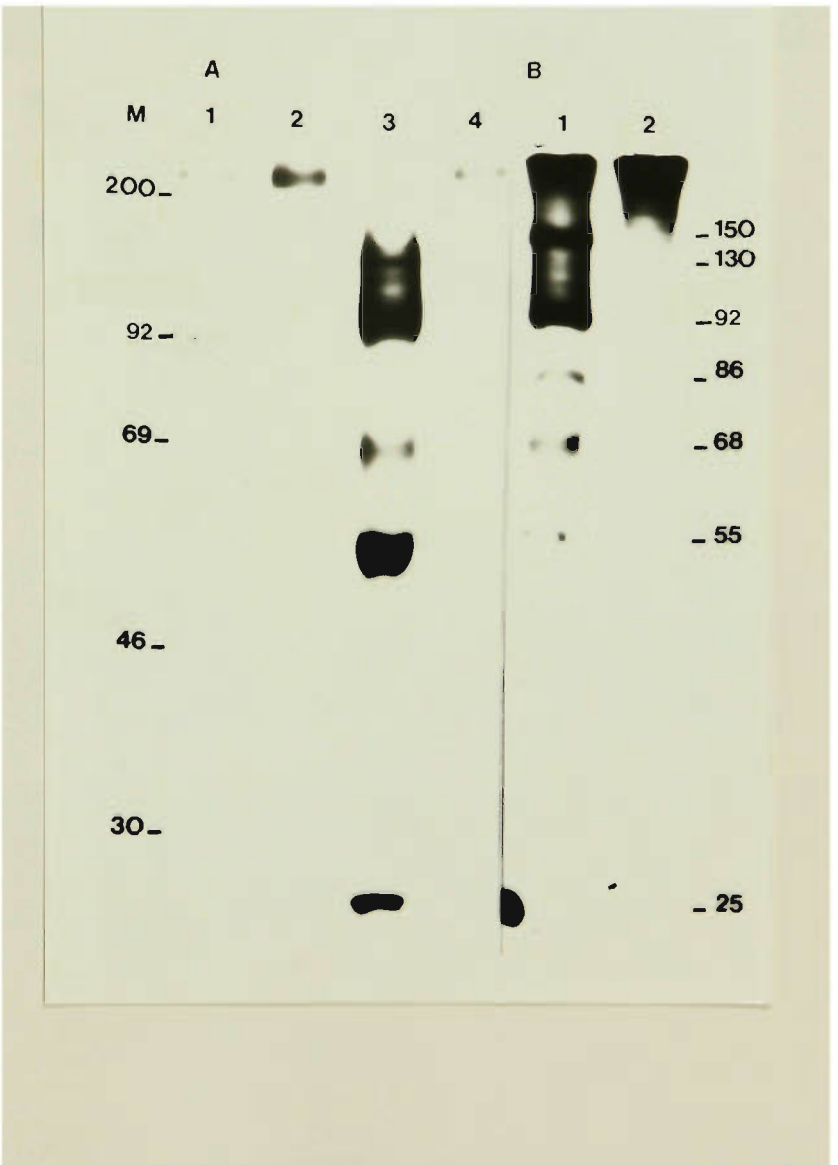


Figure 43. Autoradiograph of 10% SDS PAGE of immunoprecipitates of plasma membrane isolated from HCMV infected fibroblasts at 12h (lane 1), 48h (lane 2), and 72h (lane 3) after infection and uninfected fibroblasts (lane 4) were reacted with monoclonal antibody 15D8. Lanes B, 1 and 2 represent immunoprecipitates of infected and uninfected cell lysates extracted at 72h after infection. Molecular weights ($X10^3$) of proteins and markers (M) are indicated.

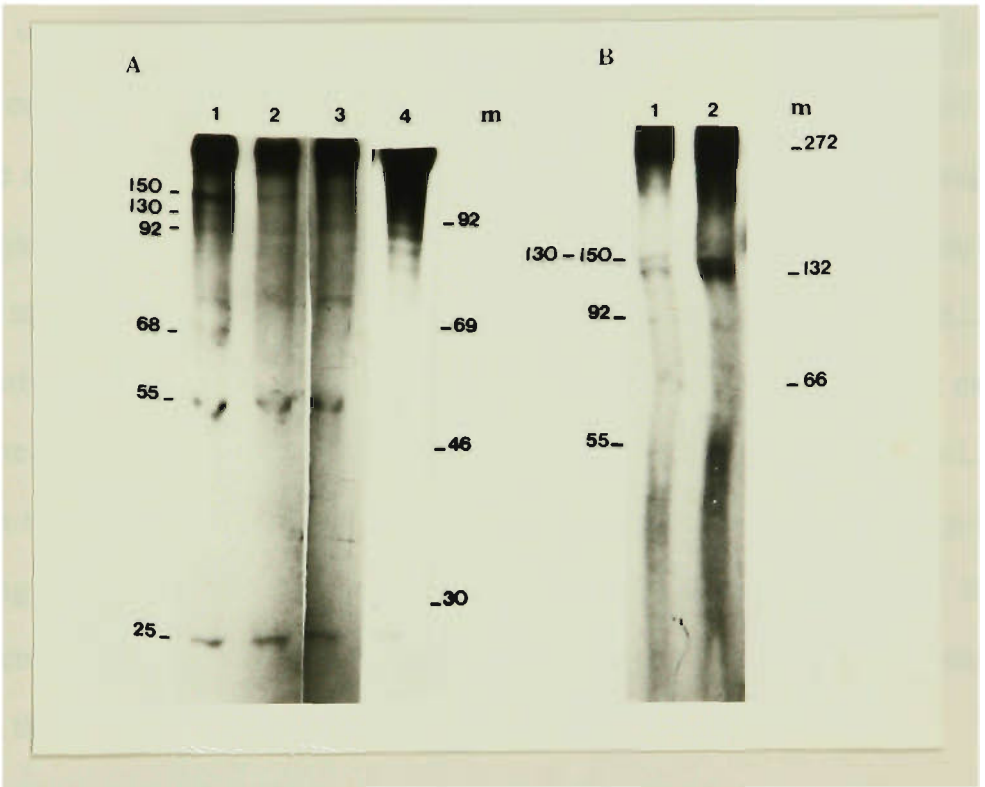


Figure 44a. Autoradiograph of 10% SDS PAGE of immunoprecipitates of plasma membranes isolated from HCMV infected fibroblasts at 12h (lane 1), 48h (lane 2), and uninfected fibroblasts (lane 3) and protein lysates from uninfected fibroblasts (lane4) reactive Mab 15D8 and exposed for a 2 week period.

Figure 44b. Autoradiograph of 7.5% non reducing SDS PAGE of immunoprecipitates of plasma membranes isolated from HCMV infected fibroblasts at 12 h (lane1) and 48 h (lane2) reactive with Mab 15D8. The proteins indicated were detected after 2 weeks exposure to the x-ray film at -70C.

after infection (Figure 44). In addition proteins were detected in the plasma membrane fractions and total cell lysates from uninfected cells.

These plasma membrane proteins detected by long term exposure at 12 h. and 48 h. after infection were of M.W. 150, 130, 68-70, 55 and 25 kDa for proteins analysed under reducing conditions. A HMWC in the region of 130 -150 kDa and proteins of 92 and 55 kDa were also detected in the plasma membrane samples isolated at 12 h. and 48 h. p.i. and analysed under non-reducing conditions. Proteins isolated at 48 h after infection appeared to have a higher affinity for Mab 15D8 than proteins from the 12 h p.i.sample or from uninfected cells. The amount of label incorporation for the uninfected cell plasma membrane fraction and the cell lysates was similar to that of the 12h p.i. samples. Although the molecular weights resemble those of proteins from the 72h p.i. sample, overlaying the autoradiographs showed that the bands in the 55 kDa and 92-94 kDa region were not the same . Further the 72h p.i. sample appears to have a slightly different profile in the 130-150 kDa region.

TableXVII.

Labelled Plasma Membrane Proteins and Cell Lysates from HCMV Infected and Uninfected Fibroblasts Immunoprecipitated with Mab's reactive with the gB Glycoprotein Complex of HCMV and analysed by Reducing SDS PAGE and Autoradiography.

	Infected		Uninfected	
Mab	m	l	m	l
15D8				
72h.p.i.	-	150	150	-
	130	130	130	-
	92-94	92-94	-	-
	-	86	-	86
	-	-	72	72
	68	68	68-70	68-70
	55	55	55	55
	25	25	25	25
48h.p.i.				
	150	ND		
	130			
	72			
	68			
	55			
	25			
12h.p.i.				
	150	ND		
	130			
	86			
	72			
	55			
	25			
Mab α 52				
	–	68		
		52		
		28		

Immunoprecipitation analysis of plasma membranes and cell lysates from HCMV infected and uininfected cells with Mab's 15D8, 7-17, α 52 and 28-2 and analysed by reducing SDS PAGE and fluorography. The molecular weights of reduced proteins ($X10^3$) are listed above.

Proteins from infected cell lysates and plasma membranes, isolated 72 h after infection were also immunoprecipitated with Mabs 7-17, CH 28-2 and α52 and analysed by reducing SDS PAGE (Tables X and XVI). Proteins from infected cell lysates of 130, 94, 68 and 58 kDa were isolated with Mab 7-17 and Mab CH 28-2 and no protein at 28 kDa and proteins of 28, 52 and 68 kDa with Mab α52 (Figure 45). The level of labelling in these samples was also much lower . No proteins were detected in the cell lysates from uninfected cells. Plasma membrane proteins of 130, 92-94 and 54-58 kDa from infected cells were also detected with both Mab 7-17 and CH 28-2(Figure 30 ; Table X). Hence the proteins immunoprecipiated with these monoclonal antibodies raised against gB are similar in molecular weight to those proteins identified with serum 4PP and anti-54 kDa antisera.

3.8. Fc Receptors for IgG on HCMV-Infected and Uninfected Fibroblasts

Plasma membranes from uninfected and HCMV-infected HFF's metabolically-labelled with L-^{35}S methionine were isolated 72h post infection and immunoprecipitated with human IgG to verify the identity of Fc receptor(s) reported to occur on the surface of HCMV-infected fibroblasts (Figure 46). One membrane protein of molecular weight 43-45 kDa was immunoprecipitated in significantly greater amounts from both uninfected and infected cells than any other proteins.

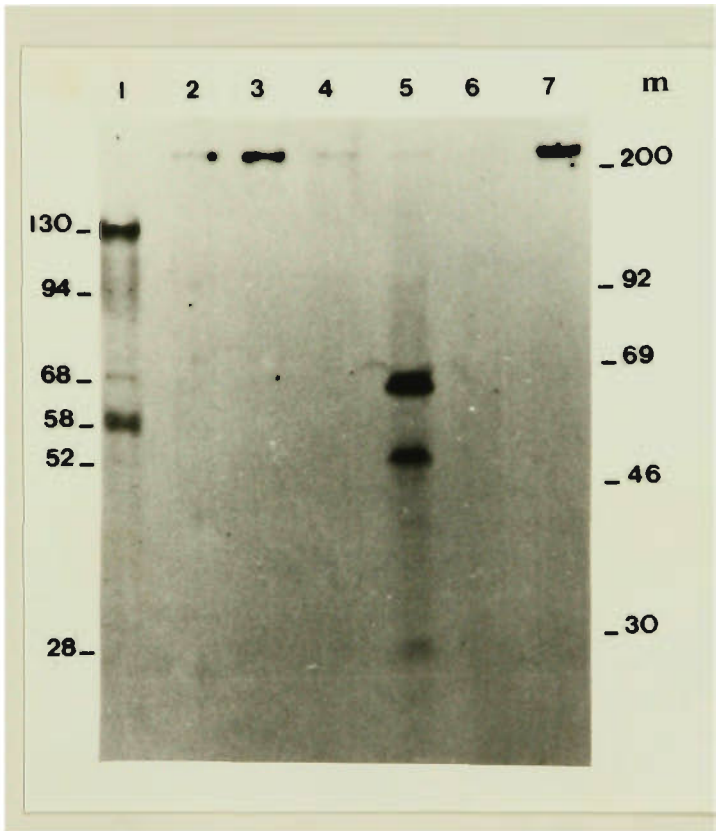


Figure 45. Immunoprecipitation of infected and uninfected cell extracts and plasma membranes preparations with Mabs 7-17 and α52. Autoradiograph of 10% SDS PAGE analysis of proteins from infected cell lysates reactive with Mab 17-7 (lane 1) and Mab α 52 (lane 5). Membranes from infected (lane 2) and uninfected (lane 3) cells and lysates from uninfected cells did not react with Mab7-17. Proteins from membranes of infected cells (lane 6) and uninfected cell lysates (lane 7) were not reactive with Mab α52.

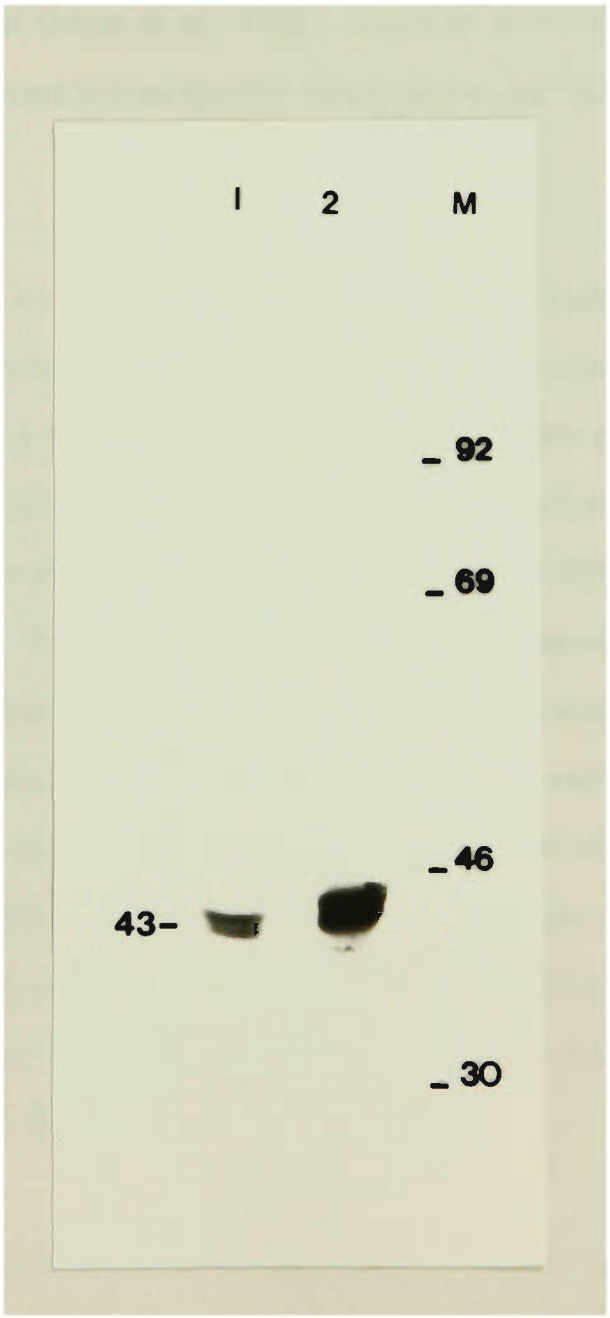


Figure 46. Autoradiograph of 10% SDS PAGE analysis of plasma membrane proteins from infected and uninfected fibroblasts immunoprecipitated with human IgG (lanes 1 and 2 respectively). Molecular weights ($X10^3$) of markers (M) and proteins are indicated.

DISCUSSION.

Infection of cells with HCMV results in significant changes in the composition, fluidity and organisation of the plasma membrane and cytoskeletal elements associated with it (Jones *et al.*, 1988). Some of these changes have also been attributed to changes in host specific factors caused by the infection (Weder and Radsak, 1983).

Previous studies have shown that HCMV infected cells contain at least 12 novel virus induced plasma membrane proteins. Six of these proteins occured with high frequency in FMA purified plasma membranes, and were of M.W. 52-54, 60-63, 70-72, 98-103, 130-133 and 260-275 kDa (Sullivan-Tailyour and Garnett, 1986). Of these, proteins of M.W. 52-54, 60-62, 70-72 and 98-103 kDa were reported to be glycoproteins. Further metabolic labelling of mannose residues allowed for the detection of proteins of M.W. 34, 43 and 145-150 kDa which were considered to be highly glycosylated. Proteins of M.W. 60-62, 70-72 and 130-133 kDa could be detected as early as 8h after infection whilst proteins of M.W. 34, 52-54, 98-103 and 145-150 kDa were detected at 24-48 h post infection, and a protein of M.W. 260-275 was only evident at late stages of infection. The synthesis of all of these proteins increased during infection and could be detected in abundance at 72-96h after infection (Sullivan-Tailyour and Garnett, 1986).

The molecular weights of some of these proteins, namely proteins of M.W.34, 52-54, 98-103, 145-150 kDa are similar to proteins reported in the virion envelope, and could represent constituents of either the gB glycoprotein complex (Cranage *et al.*, 1986; Spaete *et al.*, 1988), or the gCII glycoprotein complex (Gretch *et al.*, 1988b, 1988c). This study aimed to investigate whether or not specific proteins of the gB complex are present in the membranes of infected cells. Since the gB complex of proteins, and in particular the gp 55 component of the complex is

currently being considered for use as a subunit vaccine (Rasmussen *et al.*, 1989) it is important to know the the ultimate locality of these proteins in the infected cell. Virus induced proteins located in the plasma membrane of infected cells have previously been implicated as important in humoral as well as cell mediated immune responses such as antibody mediated cellular cytotoxity and antibody mediated cell lysis (Middeldorp *et al.*, 1986).

The characterization of plasma membrane proteins from infected and uninfected cells requires a suitable yield of undamaged membranes, uncontaminated by extraneous cytoplasmic organellar and membrane material. The FMA membrane extraction procedure allows for the selective removal of plasma membranes directly from fibroblasts grown in monolayer culture (Scher and Barland, 1972). No dissociation of the cells from the substratum into a single cell suspension, a procedure that has been shown to disrupt the plasma membrane composition, is required (Mc Clure *et al.*, 1979). The plasma membranes are stabilized before removal with zinc chloride/DMSO and FMA. Subsequent extraction of the plasma membranes by physical shearing does not result in the loss of membrane material, and the cells and cell organelles remain intact during the release of the membraneous material (Mc Clure *et al.*, 1979). The observation of adherent fibroblasts before and after release of membraneous material are in accordance with these findings (Figure 1).

Previous characterisations of plasma membranes isolated by this technique have demonstrated that the membrane fraction is essentially free from any obvious contamination with cellular organelles, except perhaps for some ribosomal material attached to the underside of the plasma membrane (Mc Clure *et al.*, 1979). It has however been shown that the efficiency of membrane release is highly variable and dependent on cell morphology (Mc Clure *et al.*, 1979). In the current study higher yields of plasma membranes were obtained from infected cells. This is possibly in

part due to the altered morphology of infected cells (Albrecht *et al.*, 1980), and an increase in protein synthesis associated with HCMV infection (Stinski 1983; Ho, 1982). In order to accommodate for the differences in protein yields between infected and uninfected cells, plasma membranes isolated from infected cells were only treated for 30 min and not 40 min during the membrane extraction step. The amount of protein loaded onto the gels for electrophoresis was also standardized (Section 2.3.1).

Although relatively high yields of plasma membrane proteins were obtained from the FMA membrane isolation procedure, a major criticism of the method, is that the FMA cross links proteins. Cross linking could result in the formation of artifacts, and it has previously been reported that the high molecular weight proteins are under represented in membrane samples isolated by FMA extraction (Mc Clure *et al.*, 1979 and Sullivan-Tailyour, 1986). Indeed in the current study, membrane preparations analysed by gel analysis and staining did not detect the high molecular weight proteins (Figure 2), metabolic labelling being required to detect such proteins (Figure 4).

A plasma membrane isolation procedure using two phase polymer systems, based on the original method developed by Brunett and Till (1971) was used as an alternative procedure to that of the FMA technique. Several modifications aimed at minimising possible artifacts during the subcellular fractionation of plasma membranes, as specified by Klockman and Deppert (1983a), were followed. The yield of plasma membrane proteins from this method of extraction and purification represented approximately 2% to 3% of the total cell protein.

The Na+,K+-ATPase assay, used to assess the purity and enrichment of the plasma membrane fraction, showed that this fraction contained the majority of the Na^+, K^+-ATPase activity (Table V).

Since the degree of confluency and passage number have been shown to affect the yield of plasma membranes from tissue culture cells (Howell, 1989), near confluent cells between passage 5 and 20 were routinely used. The use of fully confluent cells above passage 20 resulted in cell and membrane aggregation during the purification procedure, giving rise to a considerably lower plasma membrane yield. A mixture of ionic and non-ionic detergents were used to solubilise the extracted membranes. Treatment of isolated plasma membranes with NP40 alone, has been shown to solubilise only approximately half of the plasma membrane proteins (Kockman and Deppert, 1983a). Isolation of plasma membranes by the two phase polymer systems method and solubilization with both ionic and non ionic detergents is said to retain most of the antigenic epitopes of the proteins and as such is suitable for use in immunoprecipitation analysis (Klockman and Deppert, 1983a). Subfractionation of the plasma membranes into detergent soluble and insoluble fractions can be undertaken. The detergent resistant fraction of plasma membrane proteins, reported to be connected to the cytoskeleton, forms a submembraneous lamina directly underlying the plasma membrane (Mescher *et al.*, 1981). This fraction contains lipids into which certain membrane and cell surface proteins are anchored, which might provide skeletal functions for the plasma membrane (Mescher *et al.*, 1981). Such proteins could provide a basis for mediating signals into the interior of the cell. Only the detergent soluble fraction was analysed in this study.

The approach used in this study was to characterize novel HCMV induced proteins located in the plasma membranes of infected and uninfected cells by one dimensional gradient SDS PAGE and CBB staining (Figure 2). In conjunction with the latter procedures plasma membrane preparations and cell lysates, extracted from metabolically labelled HCMV infected fibroblasts and fractionated by SDS PAGE, were analysed by image subtraction analysis (Figures 3, 4, 5 and 6).

The molecular weights of novel virus induced plasma membrane proteins isolated from infected fibroblasts at 72h p.i. and identified in this study from two different plasma membrane isolation procedures after reducing SDS PAGE (Section 4.1.) are in part agreement with previously reported novel virus induced plasma membrane proteins of M.W. 52-54, 60-63, 70-72, 98-103, 130-133 and 145-150 (Sullivan-Tailyour and Garnett, 1986). Proteins of M.W. 26, 32-34, 38, 48, 54- 55, 62, 68, 72-74, 86,92-94, 105, 130 and 150 kDa were identified in plasma membrane proteins isolated with the FMA procedure from infected fibroblasts 72h post infection (Figure 6). The protein of 92-94 kDa identified in this study is synonomous with the 98-103 kDa HCMV induced novel plasma membrane protein identified by Sullivan-Tailyour and Garnett, 1986 (Personal communication, Sullivan-Tailyour). Solubilized infected cell proteins extracted from the cell residue after the removal of the plasma membranes were of M.W. 24, 38, 46, 48, 68, 72, 92, 120, 130 and 150 kDa (Figure 6). Novel virus induced plasma membrane proteins extracted by two phase polymer systems were of M.W. 46, 48, 55, 58, 62, 68, 72, 92, 130, 150, 160 and 180 kDa (Figure 3). A comparison of the proteins extracted by the FMA and two phase polymer systems methods indicated that lower molecular weight forms of virus induced plasma membrane proteins were not as readily isolated with the two phase polymer systems method.

In addition, plasma membrane proteins of 30, 46-48, 55, 62, 92 and 130 kDa were also detectable in normal host cells, although in far lesser amounts (Figures 2 and 3). This observation is in agreement with results obtained by Sullivan-Tailyour, 1986, who also showed that proteins of very similar M.W., to those identified for the infected cell could be seen in the uninfected cell in lower amounts. This result is in part due to the fact that HCMV infection does not abrogate host cell protein synthesis and could even upregulate the production of certain host cell proteins.

Monospecific polyclonal antisera was raised in rabbits against individual HCMV induced plasma membrane proteins of M.W. 54, 62 and 94 kDa and the antisera were used to assess the structural and functional association of these proteins, with proteins in the host cell plasma membrane and virion envelope, these having molecular weights similar to the constituents of both the gB and gCII glycoprotein complexes found in the virion.

All three antisera were reacted with fixed, non permeabilized fibroblasts 72h after infection. Anti-54, 62 and 94 kDa sera resulted in specific staining of the infected cells (Figures 7a, b; 8a, b; 9a, b). Examination of the cells at 400X and 1000X magnification indicated that all three antisera reacted with the plasma and nuclear membranes of infected fibroblasts. The reactivity of the 94 kDa anti-sera preparation with the cytoplasm and inclusion bodies of infected cells was noticeably stronger that with that of the anti-54 or anti-62 kDa sera. A protein in the 94 kDa molecular weight range represents the primary gene transcript of the gB complex of proteins, before any post translational modifications take place. As such it would be expected that a protein of similar molecular weight would be found in abundance in the infected cells and possibly in the plasma membranes of infected cells as well. It is therefore possible that the 94 kDa protein is a gB protein. Alternatively the 94 kDa protein detected here is the N-terminal cleavage product of the processed gB.

Indirect immunofluorescence and immunoelectron microscopy studies carried out in order to locate the position of the gB glycoprotein complex in the virus particle and infected cell with guinea pig serum reactive with the gp 55/130 proteins of the gB complex, showed that the plasma membranes of unfixed infected cells was positive from 72 hrs p.i. through to 7 days p.i. (Landini *et al.*, 1987). However these authors suggested that the plasma membranes of infected cells were only positive where virus and dense bodies budded through the membrane to form their own envelope. Two human Mabs raised against HCMV virions and reactive with proteins of M.W. 130 and 55 kDa. (Mashuo *et al.*, 1987) have been shown to bind

to the surface membrane of infected cells by immunofluorescence. However, no immunoelectron microsopy studies using these specific Mab's have been carried out to verify the findings of Landini *et al.* (1987).

The results of the current study suggest a more uniform staining of the plasma membranes of infected cells with anti-sera raised against infected plasma membrane proteins of M.W. 54, 62 and 94 kDa. Thus the question arises as to whether or not the antisera raised in this study are indeed against proteins belonging to the the gB glycoprotein complex found in the virion envelope, or whether they are related to other virion envelope complexes such as the gC II.

Since the envelope is the outermost structure of the virion, it is likely to be directly involved in the absorption and penetration of the virion into the host cell, and thus infectivity. For this reason envelope proteins are considered to be one of the most relevant groups of antigens for the induction of neutralising antibody. However the mechanisms involved in the process of envelope aquisition and as such the origin of the proteins that comprise the envelope are still not fully understood. Envelopment of the HCMV nucleocapsid was originally thought to occur at the internal nuclear membrane (Smith & de Harven, 1973) but recent electron microscope studies favour acquisition of the final envelope in the Golgi apparatus region of infected cells (Severi *et al.*, 1988) and "occasionally" at the plasma membrane (Landini *et al.*, 1987). The characterization of an additional outer osmotically fragile envelope by Farrar and Oram (1986), supports the idea that the final envelope of HCMV is indeed aquired from the plasma membrane of the infected cell upon release of the virus. Furthermore it has also been shown that during envelopment, both virally modified and unmodified normal membrane constituents are incorporated into the viral envelope. It has previously been shown that the HCMV virion envelope contains a number of glycoproteins that can induce both humoral and cellular

immunity in experimental animals (Cranage *et al.*, 1986; Furukawa *et al.*, 1984; Rasmussen *et al.*, 1985 and Gonczol *et al.*, 1986).

Plasma membranes isolated by FMA extraction from uninfected and infected fibroblasts immunoprecipitated with rabbit anti-sera raised against enveloped virions demonstrated that proteins of M. W. 96, 82, 52, 48, 42, 38, 32, 24, and 20 kDa from infected cells were reactive with anti-virus sera (Figure 14). This suggests that some of these plasma membrane proteins could be viral structural components, or modified host cell plasma membrane components that become incorporated into the virion envelope. Given these results it was decided to assess the reactivity of virion and virion envelope proteins with the antisera raised against plasma membrane proteins of 54, 62, and 94 kDa.

The compositional characterisation of HCMV proteins by SDS PAGE (Figure 15) indicated the presence of proteins that have previously been described for the virion, by Farrar and Oram, (1984). The protein composition of detergent solubilised virion envelopes and those obtained by osmotic treatment were characterised by 10% SDS PAGE analysis before immunoprecipitation with rabbit anti-sera raised against plasma membrane proteins of M.W. 54, 62 and 94 kDa of infected cells. This was carried out in order to ascertain the number and molecular weights of the proteins in these preparations as well as the affinity of these proteins to the antisera.

The protein composition of the Triton X-100 solubilized virion envelopes was similar to that obtained by Law *et al.*, (1985), with proteins of M.W. 28, 32, 54, 65 and 98 kDa (Figures 16 and 18). A major protein of 43 kDa was also obtained, and this was taken to be representative of one of the IgG Fc receptors previously identified as present in the virion envelope (Furukawa *et al.*, 1975). Immunoprecipitation of whole virions solubilised with SDS, and 2% ME with anti-

sera raised against plasma membrane proteins of M. W. 54, 62 and 94 kDa demonstrated that a virion protein of M. W. 54-58 kDa was reactive with all three antisera (Figure 19). As such, all three of these anti-sera contained antibodies that strongly react with a dominant immunogenic glycoprotein of the virus envelope (Farrar and Oram, 1984, Farrar and Greenaway, 1986). When virion envelopes obtained by osmotic shock, as described by Farrar and Oram 1984, were immunoprecipitated with antisera a major protein of 54-58 kDa was seen to react with the anti-62 and anti-54 kDa sera (Figure 20). No proteins from immunoprecipitates of these envelope preparations, with the anti-94 kDa sera, could be detected even though the anti-94 kDa sera reacted strongly with a 54 kDa protein in Triton X-100 solubilised whole envelopes (Figure 19). This could be due to the fact that only a minor protein band of M.W. 98 kDa was detected in the labelled Triton X-100 virion envelope fraction (Figure 18), and that the immunoprecipitated proteins were analysed by reducing SDS PAGE only. However the difference in the reactivity of the anti-94 kDa sera with proteins isolated from the outer and inner envelopes, could also indicate a structural and conformational difference in the orientation of the 94 kDa protein in the two membranes of the virion envelope is uncertain. These results suggest that the 54 and 62 kDa proteins are probably found on the surface of the virions, whereas the 94 kDa protein is possibly not.

Virion proteins of M.W. 54-58, 62, 68, 94, 130, 150 and 250 kDa were also reactive with anti-94 kDa sera. The structural association between proteins of 54 - 58, 62, 68, 94, 130, 150 and 250 kDa in the virion was not determined.

Virus neutralizing antibodies are known to be produced during the course of HCMV infection, and the occurence of HCMV-antibody complexes has been reported (Stadler and Ehrensberger, 1980; Rundell and Betts, 1980). Several studies demonstrating the occurence of HCMV neutralization in vitro have also been reported. HCMV proteins containing neutralizing epitopes on virions have been

primarily characterized with complement dependent and independent murine monoclonal antibodies (Britt, 1984 ; Britt and Auger, 1985; Kari *et al.*, 1987 ; Pereira *et al.*, 1982; 1984 ; Rasmussen *et al.*, 1984).

However, even in the presence of neutralizing antibodies, infectious virus can still be isolated from urine, saliva and cervical secretions (Mc Keating *et al.*, 1986; Tamura *et al.*, 1980 ; Waner *et al.*, 1977). One of the reasons put forward for the continued presence of infectious virus in the presence of neutralizing antibodies *in vivo* is that the neutralization of HCMV is inefficient. One of the factors that may contribute toward this effect is the binding of host protein β2 microglobulin to viral target proteins, thereby rendering these proteins unavailable to neutralizing antibodies (Mc Keating *et al*, 1988).

Interestingly, serum antibodies from the donor, whose lymphocytes were used in the production of these Mabs precipitated much larger amounts of the 64 kDa tegument protein than the 130 or 55 kDa proteins (Mashuo *et al.*, 1987). However, these authors confirmed that the minor protein antigens, 130 and 55 kDa of the virion, rather than the major 64 kDa antigen contain important epitopes recognised by neutralising antibodies.

Since the anti-54 kDa and anti-62 kDa sera were reactive with proteins extracted from the virion envelope by osmotic shock, it was decided to test whether they were able to neutralize the virus in vitro. To date all antibodies that neutralize HCMV have been shown to react with proteins on the virion envelope, except for one report of a neutralizing Mab raised against a normal cell protein (Michelson *et al.*, 1989).

In this study microneutralisation assays were performed with and without complement to determine whether or not the anti-54 kDa and anti-62 kDa sera

raised against infected cell plasma membrane proteins recognise neutralising epitopes of antigens located on the virion envelope. Both these anti-sera neutralised virus *in vitro* (Table VI), producing greater than 50% reduction of control plaque numbers at a m. o. i. of up to $2x10^5$ PFU per cell with IgG concentrations of 20µg and 25µg for the anti-54 kDa and anti-62 kDa sera respectively.

It was also shown that the antibodies were of sufficient affinity to neutralise over 50% of the virus without addition of exogenous complement. The addition of 2% guinea pig complement demonstrated a further decrease of approximately 10% in the number of plaques in infected cells. The presence of antibodies recognising additional epitopes with a requirement for complement, could well account for this observation. On the other hand it could also be that the epitope which is the most potent immunogen influences the complement dependence of the antibody response (Rasmussen *et al.*, 1985b). Furthermore the requirement for complement is also said to be associated with immunoglobulin subclass (Waner and Weller, 1978).

The epitope recognized by neutralizing Mab 7 - 17 reactive with gp58 of the virion envelope, used in this study to determine the antigenic relatedness of novel virus induced plasma membrane proteins with virion envelope proteins, has been mapped (Utz *et al.*, 1989). The epitope recognized by a second non neutralizing Mab, 27-28 reactive with the same protein gp58, has also been characterized by the same authors. A comparison of the epitopes recognized by these two Mab's indicates that they share overlapping sequences between amino acids 608 and 625 of the primary gp58 translation product. Competitive binding assays to HCMV of both antibodies indicated almost complete inhibition of complement independent neutralizing activity of Mab 7 - 17 by non neutralizing Mab 27 - 28.

In addition to linear epitopes an analysis of the epitopes of gp 55 of the HCMV gB glycoprotein complex, indicates that gp55 contains at least two discontinuous

neutralizing domains, mapping within a segment of 219 amino acids between residues 461 (the first amino acid of the carboxy terminal cleavage fragment of gB) and residue 681, (the last amino acid of gB) (Banks *et al.*, 1989). The proximity of domains one and two suggests that they are assembled into a major discontinuous domain and not overlapping linear domains, and that both are required to form the neutralizing epitopes. Further, all reported neutralizing antibodies and monoclonal antibodies produced against proteins of the virion envelope gB complex in animals have a complement requirement although the occurence of complement independent neutralizing human monoclonal antibodies to gB has been reported (Mashuho *et al.*, 1987). To date no correlations between the structure of the antigenic determinants and the requirement for complement can be drawn.

Interestingly it has been shown that the degree and type of sugar residue does not interfere with the antigenicity of proteins that make up the gB complex. Glycoprotein complex gp55-116 derived from two different eukaryotic expression vector systems namely, vaccinia virus and baculovirus, with markedly different types and degrees of carbohydrate modifications, were shown to elicit high levels of neutralising antibodies in mice and react strongly with human convalescent sera.

The naturally occurring humoral immune responsiveness to proteins comprising the HCMV gB complex in both normal and immunocompromised individuals has been studied by testing the reactivity of sera with isolated bands of immunoaffinity purified gB (Rasmussen *et al.*, 1989). It was shown that virus neutralising activity of patient sera did not correlate directly with the presence of gB and the authors suggest that other viral glycoproteins may contribute to neutralization of the humoral immune response.

Since it has been shown that antisera raised against HCMV induced plasma membrane proteins of 54 and 62 kDa were able neutralize virus, and react with a

protein of 54 kDa from virion envelopes, it was decided to assess the reactivity of plasma membrane proteins from HCMV infected and uninfected fibroblasts by immunoaffinity absorption chromatographic procedures.

Proteins of 54 and 62 kDa were eluted from plasma membrane preparations isolated from infected fibroblasts and passed through immunoaffinity columns prepared with anti-54 kDa IgG or anti-62 kDa IgG respectively. No proteins were eluted from plasma membrane preparations from uninfected cells. These results suggest that the antisera raised against denatured plasma membrane proteins of 54 and 62 kDa are specific for these proteins only.

Immunoblot analysis of denatured plasma membrane proteins isolated by FMA extraction from infected cells at 72 h p. i. (Table XII) with antisera raised against infected cell plasma membrane protein of 54 kDa, demonstrated binding to plasma membrane proteins 54, 32-36 and 28 kDa. This antisera also bound to the plasma membrane proteins of 44-46 and 88 kDa of uninfected cells. The reason for this cross reactivity in the light of the results obtained with this antisera from the immunoaffinity column is uncertain. It could be that proteins of 44-46 kDa represent IgG Fc receptors which were inadequately blocked by BSA .

In contrast FMA isolated plasma membrane proteins from uninfected cells immunoprecipitated with anti- 54 kDa sera and analysed directly with reducing SDS PAGE and fluorography indicated no antigenic cross reactivity with host cell proteins, whereas a similar analysis with plasma membranes from infected cells indicated the presence of three additional proteins of M.W. 42, 70 and 80 kDa which were not detected in membrane samples that had been denatured prior to the application of antisera for immunoblot analysis (Table XIII). Once again the presence of additional protein bands reactive with this antisera, could reflect either differences in the methodology employed or additional proteins reactive with the

anti-54 kDa sera not detected in denatured plasma membrane samples. Proteins of M.W. 52, 66 and 76 kDa from the supernatant fluids of infected cells also reacted with anti-54 kDa sera. The significance of these proteins is discussed further in the text.

Immunoblotting of FMA extracted plasma membranes from infected cells (Table XIII) with anti-62 kDa sera, indicated specific reactivity of the antisera for a protein of 62-64 kDa, and is in agreement with the results obtained from the immunoaffinity absorption columns. On the other hand, immunoblot analysis with antisera raised against the 94 kDa protein detected several proteins of M. W. 61-62, 54-55, 51-52, 36 and 28 kDa. That this antisera reacted with proteins in the M.W. range of 62 and 54 kDa, as well as proteins of 36 and 28 kDa, previously shown to react with anti 54 kDa sera, suggests that proteins of 54, 62-64 and 94 kDa may be antigenically related. These results could support the hypothesis that these proteins are part of the gB complex, since a primary translation product of the gB gene of 95-105 kDa undergoes extensive glycosylation and is then cleaved (Spaete *et al.*, 1988). Antigenic cross reactivity with the denatured lower molecular weight forms of this protein after glycosylation and cleavage is to be expected.

Immunoprecipitation analysis of plasma membranes from infected cells (Table XII) with the 94 kDa antisera yielded some similar proteins to those obtained from the immunoblot analysis. The additional protein bands identified with immunoprecipitation methods could represent differentially glycosylated forms of the same proteins identified in the blots or proteins where the epitopes are obscured once the proteins bind to the nitrocellulose. It is also likely that the anti-94 kDa sera contains antibodies which react with a variety of proteins and is less specific than the anti-54 kDa sera.

The question arises as to whether some of the addtional plasma membrane protein bands detected with all three antisera by immunoblot and immunoprecipitation analyses could possibly represent either endogenous IgG Fc receptors or Fc receptors induced by HCMV infection. Fc receptors have been detected on the surfaces of HCMV and HSV infected cells (Furukuwa *et al.*, 1975; Westmoreland and Watkins, 1974) and it has been suggested that coating of the infected cells with IgG or immune complexes by the Fc region of the IgG could serve to protect the cell from destruction by cytotoxic antibodies or even from cell mediated immune lysis.

Proteins of M. W. 130, 65, 50 and 38 kDa have been identified in HCMV infected cells as IgG FcR's, by virtue of the fact that isolation of these proteins from detergent extracts of HCMV-infected cells bound to immobilised rabbit IgG and ^{125}I-labelled human IgG (Xu - Bin *et al.*, 1989). Anti-HCMV antibodies were also shown to inhibit the binding of ^{125}I-labelled human IgG Fc to proteins of M. W. 130 and 50 kDa and to a lesser extent, to a protein of 65 kDa (Xu Bin *et al.*, 1989). Two other proteins at the infected cell surface that are also implicated in IgG binding are of M.W. 200 and 85 kDa. The development of FcR's has also been shown to be inhibited by 2-deoxy-D-glucose, thus indicating that the FcR's are glycoproteins (Giznou and Minamishima, 1988). The virion itself contains FcR's of M. W. 130 and 65 kDa and these proteins are said to be viral structural proteins (Xu - Bin *et al.*, 1989). Since the novel virus induced plasma membrane proteins identified in this study are of similar molecular weight to these reported Fc receptors, the possibility of some of these proteins being Fc receptors cannot be ruled out. However the only Fc receptor detected in this study,was of M.W. 43 kDa (Figure 46). This is presumed to be an endogenous Fc receptor of fibroblasts, since it also occurs on normal cells.

Proteins from the supernatant fluids of infected cell cultures of M. W. 80, 76, 66 and 18 kDa were also shown to react with anti-94 kDa sera. Some of these proteins could represent slightly altered forms of the plasma membrane proteins, reactive with anti-94 kDa sera, that are sloughed off from the cell during the course of infection. The possibility that some of these proteins could be secreted from the cell can also not be discounted. Indeed proteins from the supernatant fluid of M. W. 76 and 66 kDa were also reactive with anti-54 kDa sera. An infected cell supernatant protein of 52 kDa was recognized by both the 54 and 62 kDa antisera.

Since immunoprecipitation analyses of FMA extracted plasma membranes and immunoblot analyses indicate that there is a possible antigenic association between proteins of 54, 62 and 94 kDa, and constituent proteins of similar molecular weights occur in the gB and gC II virion envelope protein complexes, it was decided to investigate whether or not the membrane proteins under study were associated in high molecular weight complexes, not able to be indentified by reducing gel electrophoresis.

It has become well established that monoclonal antibodies and monospecific polyclonal antibodies are able to co-immunoprecipitate several antigenically and structurally related proteins, that go to make up high molecular weight protein complexes that are immunogenically important components of HCMV (Britt, 1984; Law *et al.*, 1985; Nowak *et al.*, 1984; Pereira *et al.*, 1982). The immunogenicity of the isolated virion glycoproteins has also been shown to be relatively low compared to the intact virus. This suggests that cooperative interactions between proteins that go to make up high molecular weight complexes (HMWC) are important in eliciting efficient immune responses. The three dimensional conformation of the proteins and or protein complexes is also thought to be important in the expression of some effective neutralizing epitopes. These findings also support the

identification of discontinuous domains of neutralizing epitopes of gp 55 (Banks *et al.*, 1989)

Several HMWC's have been detected in the virion envelope (Britt, 1984; Farrar and Oram, 1984; Farrar and Greenaway, 1986; Gretch *et al.*, 1988a, 1988b; Law *et al.*, 1985). To date no HMWC's of virus induced proteins have been reported in the plasma membranes of HCMV infected fibroblasts.

When labelled plasma membrane proteins isolated from infected fibroblasts by two phase polymer extraction procedures were immunoprecipitated with anti-54, anti-62 and anti-94 kDa IgG, and subjected to non reducing SDS gel conditions, a HMWC of 130 - 150 kDa reactive with all three antisera was detected (Figures 21, 23 and 25). The broad band of protein immunoprecipitated with anti-62 and anti-94 kDa IgG was resolved into three distinct HMWC 's of 130, 140 and 150 kDa for plasma membrane proteins. That all three antisera were able to recognize protein complexes in the same molecular weight range, suggests that all the protein complexes may indeed be antigenically and structurally related.

When these HMWC gel bands were cut out, reduced and re-electrophoresed, the results obtained, for the three HMWC's of 150, 140 and 130 of plasma membrane preparations immunoprecipitated with both the anti-62 kDa IgG and the anti-94 kDa IgG and the broad band of 130-150 kDa, which immunoprecipitated with the anti-54 kDa IgG, indicated that these complexes are probably structurally similar. Similar constituent reduced proteins of M.W. 130, 92-94 and 55 kDa were identified by silver staining (Figures 22, 24, 26, 27 and 28). Reduction and re-electrophoresis of the protein complex 130-150 kDa immunoprecipitated from infected cell plasma membranes with anti-54 kDa IgG suggested the presence of novel proteins of molecular weight 55 kDa and 92 kDa. Uninfected preparations although not yielding a metabolically labelled complex, did yield a protein on re-

electrophoresis and silver staining of <55 kDa. Although reduction and re-electrophoresis of complexes of 150 kDa immunoprecipitated by anti-62 kDa antisera (Figure 24), gave some hint of proteins of <55 kDa and <92 kDa, distinct bands of protein of molecular weight 55 kDa and 92 kDa were only obtained from the infected cell plasma membrane complexes, and no bands were seen at 62 kDa. The reduced proteins from the 140 kDa complex precipitated by this sera from the infected cell plasma membranes yielded similar constituent protein molecular weights. That is to say the high molecular weight protein complexes immunoprecipitated by both the anti-62 kDa sera and the anti-54 kDa sera, yielded similar constituent proteins on reduction and re-electrophoresis, suggesting that they were indeed related. Reduction and re-electrophoresis of complexes of 140 kDa and 150 kDa from infected cell plasma membrane preparations with anti- 92 kDa sera again yielded proteins of molecular weight 55-56 kDa and 92-94 kDa. No similar proteins were seen when bands in these molecular weight ranges were excised from control gels and treated similarly. As such the HMWC 140 kDa immunoprecipitated by anti-62 kDa IgG and anti-94 kDa IgG could be unique to virus infected cells. Immunoblot analyses (Table XIII), suggested that the anti-54 sera also recognizes a 28 kDa protein. This was not seen in the immunoprecipitation of complexes followed by reduction and re-electrophoresis. This may suggest that there is a 28 kDa protein with an epitope seen by the anti-54 kDa sera, but that it is not part of the complex of proteins recognized by this sera.

A precursor protein of 25-32 kDa has been identified for the glycoprotein 47-52 kDa of the gCII complex (Gretch *et al.*, 1988). Since requested monoclonal antibodies to the gCII complex could not be made available the association of a 28 kDa protein and other proteins with HMWC's in the plasma membranes of infected cells and gCII could not be assessed.

By far the two most abundant reduced proteins as seen in the current study by immunoprecipitation of infected cell plasma membrane preparations with anti-54 kDa and anti-62 kDa sera are of molecular weights 55 and 94 kDa. The subunit composition of the high molecular weight forms recognized by the 92 kDa antisera raised against plasma membrane proteins was also confirmed by the results obtained from immunoprecipitation and reducing gel electrophoresis (Figure 30). Proteins of 130, 94 and 54-58 kDa were present in plasma membrane samples immunoprecipitated with all three antisera.

An additional protein of < 54 kDa was also detected in infected cell lysates with anti-62 kDa IgG and anti-94 kDa IgG utilizing reducing gel analysis of immunoprecipiated protein complexes. Further a protein of ~ 86-90 kDa was also detected in the plasma membrane preparations of infected cells immunoprecipitated with anti-94 kDa IgG and infected cell lysates reacted with anti-94 kDa and anti-62 kDa IgG. These proteins have not been reported as constituents of the gB complex of proteins and could represent differentially glycosylated forms of the gB gene product or the presence of non specific antibodies in the antisera preparations or the interaction of Fc receptors in the immunoprecipiation reaction mixture. That minor protein bands of 130, 94 and 54-58 kDa were also detected in uninfected cell lysates immunoprecipitated with anti-94 kDa and anti-62 kDa sera supports the idea that these two antisera preparations were not as specific as the anti-54 kDa sera (Figure 30).

Proteins of the same molecular weights as those identified with anti 54 kDa IgG were also immunoprecipitated from plasma membrane preparations from infected cells with Mabs 7-17 (Britt *et al.*, 1986) and CH 28-2 (Pereira *et al.*, 1984). Both these Mabs have been shown to be reactive with the gB complex of glycoproteins located in the virion envelope. As such these results confirm the presence of virion

envelope proteins of the gB complex of glycoproteins in the plasma membranes of infected fibroblasts.

When the reduced protein components of other HMWC's immunoprecipitated from infected cell lysates and plasma membrane preparations with antisera raised against virion envelope gB constituents of 55/130 kDa were analysed, similar constituent proteins to those found in HMWC immunoprecipitated with antisera raised against HCMV induced plasma membrane proteins were found. Reduction and re-electrophoresis of HMWC's of 150 and 130 kDa and 130-150 kDa isolated from plasma membranes of infected cells with serum 6PP and 4PP (Gonczol *et al.*, 1986) respectively, indicated that the subunit proteins consisted of proteins of M.W. 94, 55 and 28 kDa (Figures 32, 33 and 35). Both serum 4PP and 6PP have been shown to recognize a HMWC of 130-150 kDa in virion envelopes, and as such this data confirms that a plasma membrane protein complex of 130-150 kDa is antigenically and structurally related to the 130-150 kDa virion envelope protein. Futhermore the reduction and re-electrophoresis of HMWC of 130-150 kDa immunoprecipitated from plasma membrane proteins and cell proteins from infected fibroblasts with Mab 15D8, reactive with the virion envelope gB glycoprotein complex, indicates a subunit composition of proteins of M.W. 94 and 55 kDa (Figure 42).

A protein of 62 kDa has not been reported as a constituent of the gB complex of proteins located in the virion envelope. However, anti-sera raised against the 62 kDa glycoprotein consistently reacted with proteins of M.W. 130, 94 and 54 kDa, although the use of this antibody in immunoprecipitation studies did not yield a 62 kDa protein. This suggests that the 62 kDa protein may be a differentially glycosylated form of the 54 kDa whose occurence varies. A protein of 60 kDa has been reported as a constituent of the gB complex in recombinant baculovirus infected cell extracts containing the gB fragment (Young *et al.*, 1989) and a protein

of 66 kDa has also been identified by immunoprecipitation of infected cell extracts with monoclonal antibodies specific for gp 130/55kd proteins (Gonczol *et al.*, 1986).

The antigenic relatedness of normal cell plasma membrane proteins, virus induced plasma membrane proteins and virion envelope proteins was further investigated with the use of monospecific polyclonal antisera specfic for the virion envelope proteins 55 and 130 kDa.

High molecular weight protein complexes of 150, 130 and 98 kDa, and 280, 220, 150 and 98 kDa were isolated respectively from plasma membranes and cell lysates of infected fibroblasts with serum 6PP (Figure 31). Since iodoacetamide, which binds specifically to free sulphydryl groups (Hjelmend *et al.*, 1978), was included in the non-reducing buffer, and buffers used in the extraction of the plasma membrane enriched fractions, the possibility of artifactual formation of high molecular weight complexes is unlikely.

The composition of these HMWC's was analysed by reduction, re-electrophoresis and silver staining of each complex (Figures 22, 24, 26, 27, 28 and 29). Reduced proteins of 54-58 kDa were evident in varying amounts for all the complexes analysed, indicating the specificity of serum 6PP for this protein. This result is consistent with a previous observation that the immunoprecipitating anti-sera is specific for this protein (Gonczol *et al.*, 1986). The range in the molecular weights of these HMWC's could be attributed to several factors. The subunit proteins that constitute the HMWC 's could bind to each other in several combinations and the degree of glycosylation of each protein could also vary considerably depending on the physiological status of the cells (Paulson, 1989). The experimental conditions employed in this study also differ to those used by Gonczol *et al.*, 1986. The extended time of electrophoresis by an additional hour once the bromophenol

marker had reached the end of the gel could also account for the isolation of intermediate HMWC forms previously not identified. Furthermore the specific elution of proteins with glycine before the addition of non reducing sample buffer and no heat treatment is likely to contribute to the isolation of less stable forms of HMWC. It could also be that these other HMWC's do not exist in the virion envelope, given that the structure of the infected host cell plasma membrane is presumed to be different.

Although serum 6PP yielded proteins from uninfected membranes as a result of excising the unlabelled protein complex in the 140-150 kDa molecular weight range from the non reducing gel and analysing it by reducing re-electrophoresis, the more specific serum 4PP did not give rise to any protein bands under similar analysis (Figures 33 and 35). The latter serum yielded reduced proteins in the 54-58 kDa range and minor proteins in the 94 kDa and 28 kDa.

The mature gB protein complex, referred to as gp 55-116 (Britt *et al.*, 1989) has been shown to be synthesized by the addition of both simple and complex N-linked sugars to a non glycosylated precursor of M.W.105 kDa. This precursor protein is then said to be rapidly glycosylated to a protein of 150 kDa and is then processed relatively slowly to form a protein of 165-170 kDa, which is subsequently cleaved to yield a mature gp55-116. The mature virion form of gp55 is said to contain 8000 daltons of N-linked sugars whilst the gp116 apparently contains 52,000 to 57,000 daltons of N-linked carbohydrates and approximately 5,000 daltons of O-linked sugars. Furthermore the sizes of the polypeptide components of the mature gp55 and gp116 were shown to be 47 kDa and 52-60 kDa respectively. The same authors have suggested that the mature fully glycosylated gp160-170 is cleaved asymmetrically to yield gp 55 and gp 116, and as such implies that gp160-170 is a heterodimer and not a homodimer as proposed by Nowak *et al.*, 1984. However it has also been proposed that gp160-170 referred to as gp 165-170 (Gretch *et al.*,

1988) is trimmed to a protein species of 138 kDa, and that this protein represents the final form of the virion gp 55-116 complex.

In the current study the HMWC's of 280 and 150 kDa in the plasma membrane isolated from infected cells and 150 kDa in the infected cell lysate contained higher amounts of the 54-58 kDa protein as well as the 24-28 kDa protein than any of the other HMWC's immunoprecipitated with serum 6PP antisera, possibly indicating a higher affinity of the antisera for the aforementioned protein complexes. One explanation for the occurence of molecular weight forms of greater than 150 kDa, could be the formation of multimers of high molecular weight complexes of proteins. For example, the 280 kDa HMWC's could represent a dimer of the 150K complex within the membranes of infected cells. It has also been previously suggested that HMWC's composed entirely of the 55 kDa protein can be formed (Rasmussen *et al.*, 1985). Alternatively some of the less frequently isolated HMWC 's could represent less stable forms or intermediate forms of the HMWC 150 kDa.

Only one HMWC of 130-150 kDa was identified in plasma membranes isolated 72h after infection and immunoprecipitated with serum 4PP (Figure 34). This complex consisited of a major 55 kDa protein with minor proteins of 130, 94 and 68 kDa. In contrast the unlabelled HMWC's of 130-150 kDa immunoprecipitated by serum 4PP from plasma membranes from uninfected cells contained only one protein of molecular weight < 55 kDa, and therefore indicates that serum 4PP does detect unique virus proteins in infected cells. The same result was obtained when HMWC's of 130-150 of plasma membrane proteins from infected and uninfected cells reactive with Mab 15D8 were reduced and re-electrophoresed.

Time course analysis of plasma membrane preparations, extracted from metabolically labelled infected and uninfected cells at 12h and 48h post infection

(Figure 37), and immunoprecipitated with serum 4PP indicated the presence of HMWC's of 250 and 130-150 kDa in both the 12 h and 48 h p.i.samples. A greater abundance of labelled protein in the HMWC's of the 48 h sample was evident. Reduction and re-electrophoresis of labelled HMWC's from plasma membranes isolated at 12 h and 48 h p.i. and unlabelled HMWC of 130-150 immunoprecipitated from uninfected cell plasma membrane extracts, gave rise to proteins of M.W > 130, 94, 86 and 55 kDa in all three samples. A labelled HMWC of 250 kDa, which was consistently isolated from uninfected plasma membrane samples with both sera 6PP and 4PP also contained reduced sub unit proteins of 130, 94 and 55 kDa. Furthermore a host cell plasma membrane HMWC of >500 kDa reactive with serum 6PP contained reduced proteins of 150, 130, 92 and 55 kDa.

However the HMWC of 130-150 kDa from infected cells at 12h and 48h after infection also yielded proteins of 130 kDa and 94 kDa. These were not detected in the unlabelled HMWC of 130-150 kDa from uninfected cells. Further a protein in the molecular weight region of 55 kDa was also less abundant in uninfected cells than the 55 kDa protein from the two complexes found in the infected cells. This could be due to the production of specific virus proteins of 130 and 92 kDa which are associated with the HMWC 130-150 kDa. It could also be that these virus specific proteins attach to host cell proteins making HMWC's of 250 and 500 kDa. These results suggest that there may be proteins specific for the gB complex as well as some host complexes, of molecular weights greater than 130-150 kDa that react with antisera specific for virion envelope proteins 55/130 kDa in the plasma membranes of normal cells..

Although it is well established that sera 6PP and 4PP react with the virion envelope gB proteins of M.W. 55 and 130 kDa, the fact that proteins of similar molecular weights are also detected when HMWC's of plasma membrane preparations from

host cells are reduced, re-electrophoresed and silver stained should not be discounted.

The cross reactivity of antisera with host plasma membrane proteins detected in the current study can possibly be attributed to different types of antibodies within the monospecific polyclonal mixture of antibodies, raised either against virion induced proteins found in the the host cell plasma membrane, or in virion envelopes which have possibly been aquired from host cell membranes. Hence it was decided to use the monoclonal antibodies employed in the classification of the virion gB complex of proteins to resolve whether or not the proteins detected in the host cell were due to non specific antisera, or whether the host cell itself contained epitopes similar to those found in the virion envelope that react with the neutralizing Mabs.

Comparisons of immunoprecipitated plasma membrane preparations from HCMV infected cells, isolated late in infection and separated under reducing and non-reducing gel conditions, indicates that an antigenically related complex of proteins recognised by Mab 15D8 in the virion envelope also exists in the plasma membranes of infected cells (Figures 41 and 43). Reduced proteins of 94 and 55 kDa were precipitated in abundance from the enriched plasma membrane fraction with Mab 15D8. Proteins of 25, 68-70 and 130 kDa were also detectable when analysed directly by reducing SDS PAGE. A protein of 150 kDa was not evident when immunoprecipitated plasma membrane preparations were analysed directly by reducing SDS PAGE and fluorography. This observation is in part agreement with van der Voort *et al.*, (1989), who suggested that the 150 kDa was only a minor species of the gB complex in the plasma membranes of infected cells. The 150 kDa protein is however part of the HMWC of 130-150 kDa reactive with Mab 15D8, and detected by non reducing analysis.

Proteins of higher molecular weights were detected in the infected cell lysates reacted with Mab 15D8 and analysed under reducing gel conditions, possibly indicating an abundant occurence of the HMW forms in the infected cell. Two major bands at 150 and 92 kDa were present, and the 55 kDa protein was just detectable. These results possibly reflect what is known about the processing and cleavage of the gB protein, and indicate that the cleaved 55 kDa protein is indeed located in the plasma membrane, and is formed from a high molecular weigh- intracellular precursor of 150 kDa, which is not readibly detected in plasma membrane fractions analysed directly by reducing SDS PAGE. Furthermore it has also been reported that a protein of 150 kDa of the gB complex of proteins referred to as gp 150 is relatively stable in the infected cell, is transported through the Golgi apparatus by a saturable transport mechanism and occurs in high concentrations (Britt *et al.*, 1989).

The initial autoradiograms of immunoprecipitated plasma membrane preparations isolated at 12h and 48h p.i. with Mab 15D8 analysed under reducing and non reducing gel conditions indicated that no proteins were present at these times post infection and that there was no reaction with control cells. However, prolonged exposure of the autoradiograms (2 weeks) to detect minor protein species indicated that a range of proteins in the molecular weight regions of 25, 55, 130 and 150 kDa are present at 48h and 12h after infection (Figure 44a). These are not dissimilar to proteins found in control cells. HMWC's of 130-150 kDa were also detected in the unreduced immunoprecipitated plasma membrane extracts isolated at 12 h and 48 h after infection (Figure 44b). When the gels were exposed for two weeks higher amounts of immunoprecipitated protein were detected in the 48h p.i. sample than that of the 12h p.i. sample. This result is similar to the one obtained for immunoprecipitated proteins at 12h and 48h p.i. with serum 4PP.

That the host cell contains proteins that are antigenically related to the virion structural proteins could be of importance with regard to the immunopathobiology of HCMV. Patients with HCMV infections frequently exhibit high titres of serum antibodies specific for HCMV proteins. Furthermore it has also been shown that individuals with HIV frequently show progressive disease despite increasing titres of HCMV specific serum antibodies (Simmons, 1977). It is possible that antibodies which cross react with host cell antigens could contribute to the development of autoimmune sequelae associated with infection (Farrell *et al.*, 1989).

Several neutralizing and non neutralizing Mab's raised against murine cytomegalovirus (MCMV) have been reported to cross react with both MCMV structural proteins and host cell proteins (Farrell *et al.*, 1989). These Mab's react with either nuclear or cytoplasmic antigens in MCMV infected and uninfected cells. Cross reactivity between neutralizing 144/98/92 Kd MCMV antigen (proteins said to be the murine equivalent of the gB complex of proteins) and host cell proteins were shown to occur. The same Mab also reacted with a 200 Kd cytoplasmic protein in infected and uninfected cell lysates, and purified extracellular virions.

A complement independent HCMV neutralizing monoclonal antibody was shown to react with normal cell plasma membrane proteins of human origin (Michelson *et al.*, 1989). This Mab, Mab N2 immunoprecipitated a glycoprotein of M.W. 94 kDa from both uninfected and HCMV infected cells and an additional protein of 34 kDa which was not structurally associated by disulphide linkages to the 94 kDa protein. This data illustrates yet another example of the aquisition by HCMV during virus assembly of a normal cell protein with a neutralizing epitope. It would be interesting to determine whether or not this Mab reacts with any of the HCMV induced novel plasma membrane proteins isolated in this study.

More recently sequence homology and immunological cross reactivity between products of the HCMV genome and normal cellular molecules have been reported. Of consequence to the immunoregulation of HCMV infection has been the identification of a HLA Class I related gene seqence (Beck and Barrell, 1988).

Further, a report by Revello *et al.*, (1986) showed that primary HCMV infection can be unambiguously diagnosed by the appearance of IgM directed against normal cell membrane antigens in the sera of individuals undergoing their first encounter with HCMV. The sequence of the variable regions of the heavy and light polypeptide chains of a human neutralizing IgG1 monoclonal antibody is homologous to IgM rheumatoid factors of the Wa idiotypic family (Newkirk *et al.*, 1988). This serological and structural similarity of a major idiotypic family of rheumatoid factor antibodies and the anti- HCMV antibody indicates a possible association at the level of the immune response between HCMV and rheumatoid arthritis.

Immunoprecipitation of enriched purified plasma membranes preparations and cell lysates isolated from metabolically labelled HCMV infected fibroblasts at 72h after infection with Mab 7-17 and analysed by reducing SDS PAGE only, demonstrated the presence of the gB component proteins within the infected cell and in the plasma membranes (Figures 45 and 30 respectively). Mab 7-17 was strongly reactive with proteins of 130 and 58 kDa and mildly reactive with a broad protein band between 92-120 kDa and a protein of 68 kDa. These molecular weights are in accordance with those of proteins isolated from the virion envelope by Britt *et al.*, (1986) except for the 68 kDa protein, which has not been reported for the virion envelope.

Immunoprecipitation of infected cell lysates and plasma membranes isolated from infected fibroblasts with Mab α52 did not indicate the presence of the gB complex of proteins in the plasma membranes. However two proteins of 68 and 52 kDa

were immunoprecipitated in abundance from infected cell lysates and minor components of 92, 50, 28 kDa were also detectable. Proteins of 68 and 28 kDa were not reported to occur in the virion envelope (Farrar and Greenaway, 1986) These results indicate that the 68 and 28 kDa proteins may be restricted to infected cells. Interestingly when the same monoclonal antibody was reacted with infected cells directly, immunofluorescence studies suggested that the gB proteins were present on the surface of the cells (Law *et al.*, 1986). It is possible that the lack of reactivity of Mab α52 with plasma membrane proteins of infected cells was due to a loss in antibody activity before use as it had been stored for sometime before use in the analysis of membranes.

The reactivity of Mabs 17-7 and 15D8 for protein antigens in infected cell lysates as analysed by immunoprecipitation and reducing SDS PAGE is identical. However, reactivity with Mab α52 under reducing conditions indicates that it recognises a protein of a lower molecular weight. It is highly likely that this protein is a less glycosylated form of the 55 kDa protein which has been estimated to be in the molecular weight range of 48-52 kDa (Britt *et al* ., 1989 and Spaete *et al* ., 1989).

In conclusion , this study has shown that novel plasma membrane proteins of 54, 62 and 94 kDa are structurally associated with a HMWC of 130-150 kDa, which is antigenically similar to a HMWC of the gB complex of glycoproteins located in the virion envelope. Antisera raised against all three proteins were reactive with the virion envelope protein 54-58 kDa. Further the antisera raised against reduced components of 54 and 62 kDa of the plasma membrane of infected cells are able to effect neutralization of the virus in the absence of complement. Monospecific polyclonal antisera raised against HCMV infected cell plasma membrane protein 54 kDa, serum 4PP and Mabs 15D8 and 7-17 are specific for HMWC of 130-150 kDa of infected cell lysates and plasma membrane preparations, which are comprised primarily of reduced proteins of 92-94 and 54-58 kDa. Further a comparison of

proteins which are reactive with Mab 15D8 from ^{125}I labelled infected cells and purified enriched plasma membranes isolated by the polymer systems method indicated that proteins of 130, 92-94 and 55 kDa are membrane proteins and are exposed on the surface of infected cells (Roig Farran, personal communication).

It was also shown that a range of proteins similar in molecular weights to some of the gB virion envelope proteins are located in the plasma membranes of infected cells and that proteins of normal uninfected fibroblasts cross react with Mabs specific for the virion gB glycoprotein complex. The immune consequences of normal proteins that react with Mabs specific for immunogenic virion proteins should be thoroughly investigated, in that the reactivity of normal cell proteins with antibodies "specific" to viral proteins could possibly contribute to non viral immune consequences of HCMV infection. Further, if gB proteins are to be used as a sub unit vaccine, the presence of similar proteins in normal cells is most likely to interfere with the efficacy of the immune response and hence protection against HCMV infection.

APPENDIX.

Cell Culture.

Phosphate buffered saline (PBS) pH 7.0.

(calcium and magnesium free)

NaCl	8.0g	KH_2PO_4	0.2g
KCl	0.2g	$Na_2HPO_4.12H_20$	2.89g

in 1 litre distilled water.

Hanks balanced salt solution (10x concentrate).

Solution A

NaCl	80g
KCl	4g
$MgSO_4.7H_20$	2g
Distilled water	400ml.

Add solution A to B

Solution B

$CaCl_2$	1.4g
Distilled water	100ml

Solution C.

KH_2PO_4	0.6g	Phenol red	0.2g
Na_2HPO_4	0.6g	$NaHCO_3$	3.5g
Glucose	10.0g	Distilled water	500ml

Autoclave A + B and C separately.

Combine aseptically and refrigerate

Mycoplasma detection with DAPI.

(4-6-diamine-2-phenyl-indole dihydrochloride)

Dissolve DAPI crystals in water to a concentration of 5 µg/ml to give a 50 x concentrated solution. Freeze 0.2ml aliquots at -20°C.

Test for monolayer cultures.

1. Grow fibroblasts to a density of 50% on cover slips.
2. Make 0.2ml stock solution to 10ml with absolute methanol.
3. Pour off the medium from the cells and wash 2 x with PBS.
4. Wash with the DAPI methanol solution.
5. Cover the cells with DAPI solution and stain for 15 min at 37°C.
6. Pour off the solution and wash with methanol.
7. View under the microscope 100 x oil immersion.

Dextran-polyethylene glycol aqueous two-phase system.

Stock solutions.

22g of Dextran 500 in 100ml gd water (22%)

30g of Polyethylene glycol 6000 in 100ml gd water (30%).

Mix the following reagents.

100g of 22% Dextran 500

51.5g of 30% PEG 6000

45ml of gd water

160ml, 0.22M Phosphate buffer (with sodium salts) pH 6.5

40ml, 5mM $MgCl_2$

Add DTTand EGTA to final concentrations of 5mM and 1mM respectively.

Mix in a separatory funnel and allow the two phases to settle at 4°C overnight. Collect the two phases.

KM Buffer (pH 6.2).

10mM NaCl
1.5mM $MgCl_2$
5mM DTT
1mM EGTA
10mM MOPS
1% (v/v) Apronitin
2mM PMSF
5ug/ml DNase
0.2M Iodoacetamide

Lysis Buffer (pH 6.5).

5mM $MgCl_2$
5mM DTT
1mM EGTA
10mM MOPS
1% Apronitin
2mM PMSF
5mg/ml.DNase
o.2M Iodoacetamide

Phosphate Assay.

Stock Solutions.

1. **2N Acetic Acid.**

   46ml glacial acetic acid (17.5N) into

   354ml gd H_2O.

2. **Copper Acetate pH 4.0.**

   1g $CuSO_4.5H_2O$

   18.4g Na.Acetate.$3H_2O$

   400ml 2N Acetic Acid.

3. **Ammonium Molybdate.**

   5g NH_3 molybdate

   100ml gd H_2O.

4. **ELON (p-methylaminophenol sulphate).**

   5g Na_2SO_3

   100ml gd H_2O

   Add: 2g ELON.

Reagents for SDS PAGE Slab Gels.

Stock solutions

A. Acrylamide/Bis (30% T.2,67% C).

146g acrylamide (29.2g/100ml)

4g N'N'-Bis-methylene-acrylamide (0.8g/100ml)

Make to 500ml with distilled water. Filter and store at 4°C in the dark (30 days maximum).

B. Resolving gel buffer.

1.5M Tris-HCl, pH 8.8

54.45g Tris base (18.15g/100ml)

~150ml distilled water

Adjust to pH 8.8 with 1N HCl. Make to 300 ml with distilled water and store at 4°C.

C. Stacking gel buffer.

0.5M Tris-HCl, pH 6.8

6g Tris base

~60ml distilled water

Adjust to pH 6.8 with 1N HCl. Make to 100ml with distilled water and store at 4°C.

D. 100% SDS.

Dissolve 10g SDS in water with gentle stirring and bring to 100 ml with dH_2O.

E. Sample buffer SDS reducing buffer (store at room temperature)

Distilled water	4.0ml
0.5M Tris-HCl, pH 6.8	1.0ml
Glycerol	0.8ml
10% (w/v) SDS	1.6ml
2β-mercaptoethanol	0.4ml
0.5% (w/v) Bromophenol blue	0.2ml
	8.0ml

F. 5X Electrode (Running) Buffer, pH 8.3

Tris base	45g	(15g/l))
Glycine	216g	(72g/l)
SDS	15g	(5g/l)
to 3 litres with dH_2O		

Store at 4°C. Warm to 37°C before use if precipitation occurs.

Gel Preparation: Linear SDS PAGE Slab Gels.

A. Acrylamide Plug.

19% Acrylamide/Bris (30/0.8) stock	3ml
TEMED	10µl
10% Ammonium per sulphate	2ml

Stacking Gel Preparation - 4% gel, 0.125M Tris, pH 6.8.

Distilled water	6.1ml	12.2ml
0.5M Tris-HCl, pH 6.8	2.5ml	5ml
10% (w/w) SDS	100µl	200µl
Acrylamide/Bis (30% stock	1.3ml	2.6ml
10% ammonium persulfat	50µl	100µl (0.5%)
TEMED	10µl	20µl (0.1%)
TOTAL STOCK MONOMER	10ml	20ml

Separating Gel Preparation 0.375M Tris, pH 8.8.

	12.0%	10.0%	7.5%	5.0%
Distilled water	33.5	21.675	48.5	29.175
1.5M Tris-HCl, pH 8.8	25.0	5.625	25.0	5.625
10% (w/v)SDS stock (store at room temp.)	1.0	400µl	1.0	400µl
Acrylamide/Bis (30% stock)	40.0	1.5	25.0	7.5
10% ammonium persulfate (fresh)	500µl	225µl	500µl	225µl
TEMED	50µl	15µl	50µl	15µl
TOTAL MONOMER	100ml	45ml	100ml	45ml

Gradient SDS PAGE Gels.

(a) 10% - 20%

(b) 7.5% - 15%

Gradient SDS Page.

	(a)		(b)	
	10%	20%	7.5%	15%
Distilling water	7.2		8.425	4.725
1.5M Tris-HCl pH 8.8	4.5	4.5	1.925	1.925
50% Glycerol	0.36	1.62	0.25	1.0
10% (w/v) SDS Stock	150µl	-	150µl	-
Acrylamide/Bis (30:008) stock	6.075	11.925	3.75	7.5
10% Ammonium persulphat	67.5µl	67.5µl	67.5ml	67.5µl
Temed	14µl	14µl	7.5µl	7.5µl
Total Monomer	20m	20ml	15ml	15ml

10% and 20% stocks or 7.5% and 15% stocks were placed in a gradient mixer and poured into vertical slab gels.

Destaining Solution:

A: Electrophoretic Diffusion.

30% Methanol	150ml
10% Acetic acid	50ml
gd Water	300ml
	500ml

B : Passive Diffusion:

12.5% Isopropanol	25ml
10% Acetic Acid	20ml
gd Water	155ml

Silver Staining of Gels.

Soln.A. 0.8g $AgNO_3$ in dH_2O (ml)

Soln.B. 21ml 0.3670 NaOH
1.4ml NH_4OH (Conc.)

Soln.C. Add A to B dropwise. If a brown precipitate occurs, add a few
Make up to 100ml with dH_2O.

Soln.D. 2.5ml 1% citric acid
0.25ml 37% Formalin
Make up to 500ml with dH_2O

Destain:

Soln.E. 1.5M Sod. triosulfate (23.7g/100ml)

Soln.F.

0.15M $CuSO_4$	(3.75g)	Make up to 100ml. If
0.6M NaCl	(3.5g)	a precipitate occurs,
0.9M NH_4OH	(6ml)	add more NH_4OH.

Combine equal vols E + F. Mix until gel destains.

SDS PAGE Molecular Weight Protein Standards.

1. High Molecular weight protein standards (Pharmacia).

Protein	Mol.Wt.	Subunit Mol.Wt.
Thyroglobulin	669,000	330,000
Ferritin	440,000	18,500
		(220,000)
Catalase	232,000	60,000
Lactate Dehydrogenase	140,000	36,000
Albumin	67,000	67,000

2. Low Molecular weight protein standards (Pharmacia).

Protein	Mol.Wt.
Phosphorylase b	94,000
Albumin	43,000
Carbonic anhydrase	30,000
Trypsin inhibitor	14,400

The lyophilised mixtures (100mg of each protein) of either high molecular weight protein standards or low molecular weight proteins were reconstituted with 100ml of electrophoresis buffer and boiled for 3 mins.

Sigma Non-denatured protein molecular weight markers.

Protein	Mol.Wt.
Urease	545,000 (hexamer)
Albumin (bovine serum)	272,000 (trimer)
	132,000 (dimer)
	66,000 (monomer)
Albumin (chicken egg)	45,000
Carbonic anhydrose (bovine erythrocytes)	29,000
Lactalbumin (bovine milk)	14,200

Pre-stained protein standards (Bio-Rad).

Protein	Mol.Wt.
Phosphorylase b	130,000
Bovine serum albumin (BSA)	75,000
Ovalbumin	50,000
Carbonic anhydrase	39,000
Soybean trypsin inhibitor	27,000
Lysozyme	17,000

To visualise the standards during electrophoresis 10ml/ml was loaded onto slab gels.

Rainbow^{TM} Protein Molecular Weight Markers: ^{14}C-labelled (Amersham).

Protein	Mol.Wt.	Colour
Myosin	200,000	Blue
Phosphorylase b	92,500	Brown
Bovine serum albumin	69,000	Red
Ovalbumin	46,000	Yellow
Carbonic anhydrase	30,000	Orange
Trypsin inhibitor	21,500	Green
Lysozyme	14,300	Magenta

10µl (1.4kBq) of the above mixture of ^{14}C-labelled protein standards was added to an equal volume of electrophoresis buffer, boiled at 100^{o}C for 1 min, and loaded per well.

^{14}[C]-methylated molecular weight markers (Amersham).

Protein	Mol.Wt.
Myosin	200,000
Phosphorylase b	92,500
Bovine serum albumin	69,000
Ovalbumin	46,000
Carbonic anhydrase	30,000
Luspzyme	14,000

Ammonium Sulphate Precipitation.

1. **Borate Buffer (0.1M, pH 7.4).**

Disodium tetraborate	9.45g in 25ml gd H_2O.
Boric acid	24.73g in 4l gd H_2O

Add 115ml of borate solution to 4l of boric acid solution until pH is 7.4

(2) **Borate Saline Buffer**

Boric acid	6.18g
Sodium tetraborate	9.54g
NaCl	4.38g

Make up to 1l with gd water.

(3) **Preparation of Dialysis Tubing.**

Dialysis tubing (mol.wt. 12kd to 14kd) was steamed 10 mins in 0.1mM EDTA containing 2% $NaHCO_3$. Over a period of ~ 5h the tubing was rinsed at least five times in glass distilled water. Following this the tubing was again steamed in 0.1mM EDTA containing 2% $NaHCO_3$ and stored in this solution at 4°C.

Reagents for Affinity Chromatography.

(1) Protein-A-Sepharose.

(2) Cyanogen Bromide Activated-Sepharose.

Antiserum purification : Protein A-Sepharose Affinity Chromatography.

A. Reagents.

1. **Buffer A:** (pH 8.0)
   0.16M boric acid
   0.012M NaCl
   0.0025M NaOH
   0.1mM phenylmethylsulfonyl fluoride (PMSF)
   0.02% NaN_3

2. **Buffer B:** (pH 3.0)
   0.1M glycine-HCl
   0.1mM PMSF
   0.02% NaN_3

B. Protein A Column

10ml slurry of protein A-sepharose Cl-4 packed in K9/15 (0.9 x 15cm) column, equilibrate with buffer A.

Antigen Purification-Cyanogen Bromide Activated-Sepharose Affinity Chromatography.

REAGENTS:

1. **Coupling Buffer, pH 8.5**

   0.2M $NaHCO_3$

   0.5M NaCl

2. **Acetate Buffer, pH 4.0**

   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)

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

3. **Elution Buffer, pH 11.5**

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

Removal of Triton X-100 from protein samples (Holloway, 1973).

Triton X-100 causes diffuse leading and trailing edges of gps in sodium deoxycholate gels, consequently excess Triton X-100 was removed using Bio-Beads SM-2. Bio-Beads SM-2, a neutral porous styrene-divinylbenzene copolymer, strongly adsorb Triton X-100.

Adsorption onto Bio-Beads SM-2 (Bio-Rad, USA).

Washing of copolymer beads

To 30g Bio-Beads SM-2 200ml methanol was added. The mixture was stirred for 15min and the copolymer beads collected onto a scinterred glass filter and washed with a further 500ml of methanol. The beads were not allowed to dry but washed immediately with 1000ml glass distilled water. The moist beads were then slowly washed with a further 2000ml of glass distilled water. The beads were stored under water until required. The above manipulations were performed at room temperature.

Procedure for removal of Triton X-100

Moist copolymer beads (0.6g) were added to a 2ml protein sample containing 10mM potassium phosphate pH 7.2 and various amounts of Triton X-100. The sample was gently agitated at 4°C for 2h at 4°C. The protein sample was aspirated into Nunc vials. Protein and Triton X-100 determinations were performed. (Triton X-100 was estimated from its adsorbance at 275nm.)

Reagents for Immunoprecipitation.

Extraction Buffer pH 7.2.

0.05M Tris-HCl

0.15M NaCl

1% Sodium deoxycholate

0.1% SDS

1% NP-40

5ug/ml DNase

2mM PMSF

1% (v/v) Apronitin.

Buffer A pH 7.3

10mM Tris-HCl

1mM $CaCl_2$

0.15M NaCl

1% Triton X-100

2mM DMSF

1% Ethanol

Western Blots

Transfer Buffer

Trizma Base	9g	25mM
Glycine	42g	192mM

20% methanol (v/v)

Make up to ml with gd water

Tris buffered Saline (TBS)

20mM Tris	4.84g
500mM NaCl	58.48g

Make up to 200ml with gd water

Adjust pH to 7.5 with IN HCl.

Tween-Tris Buffered Saline (TTBS)

0.625ml of Tween 20/250ml of TBS

Blocking Solutions

3-5% BSA in TBS or 5-10% skimmed milk.

Antisera Buffer

1% BSA in TBS or 1% skimmed milk.

Colour Development

0.6ml of 0.3% chloro-1 napthol in methanol

5µl of H_2O_2

Make up to 10ml with gd water

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