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CELL SURFACE CHANGES INDUCED BY VACCINIA VIRUS

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Presented for the degree of Doctor of Philosophy
in the Faculty of Science, University of Glasgow.

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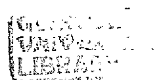

Imagination is more important than knowledge; knowledge is limited, imagination embraces the world, stimulating progress, giving birth to evolution.

Albert Einstein

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SUMMARY

Virus-specified antigens induced in the membranes of infected cells have been implicated as primary targets in the recognition and destruction of virus-infected cells by the immune system of mammalian hosts.

Antigenic changes induced in the plasma membranes of vaccinia-infected HeLa cells 2 h post-infection were detected by immunofluorescence, immune haemadsorption, and to a lesser extent by complement-fixation and complement-mediated cytolysis. Cytopathic effects (cell rounding) were seen prior to viral DNA replication and also when this was blocked. Other changes occurred at the cell surface later in infection when turkey erythrocytes and concanavalin A were bound by cells. There was no evidence for an overall increase in mucopolysaccharide synthesis in the form of sialic acid. Quantitative spectrophotometric assays for haemadsorption, immune haemadsorption and concanavalin A binding were developed and the effect of varying growth or reaction conditions on the expression of vaccinia haemagglutinin at the surface of infected cells was investigated. Vaccinia cell surface haemagglutinin (VHA), detected by the binding of turkey erythrocytes, was synthesised around 10 h post-infection, even under conditions of low multiplicity of infection, and was closely coincident with the appearance of infectious virus.

Of seven different cell lines tested, haemadsorption was greatest in HeLa cells and least in L-929 cells. Other red cell species were bound to infected cells to a lesser degree than turkey cells.

Haemadsorption occurred within the physiological and alkaline pH ranges and at temperatures between 20°C and 56°C. Haemadsorption was enhanced by pre-treatment of cells with trypsin or by infecting the cells in the presence of trypsin. Hyperimmune rabbit anti-vaccinia γ-globulin prevented haemadsorption (titre 1/100) and also specifically displaced turkey red cells adsorbed to infected HeLa cells. Glutaraldehyde fixation of infected cells or susceptible red blood cells also prevented haemadsorption. An increase in concanavalin A binding sites on vaccinia-infected cells was demonstrated, distinct from binding sites for turkey red cells.

Plasma membranes were isolated from uninfected, or vaccinia-infected HeLa cells from monolayer cultures. The cells were swollen in low molarity buffer, gently homogenised and plasma membranes purified by differential and sucrose density gradient centrifugation. The membrane fraction was characterised by phase contrast microscopy, polyacrylamide gel electrophoresis, specific uptake of ^{3}H-L-fucose into membrane glycoproteins, and by the presence of the marker enzymes, potassium-dependent phosphatase and phosphodiesterase. The absence of the marker enzymes, NADH diaphorase (oxidase) and β-glucuronidase, which are not plasma-membrane associated, was also noted.

In immunofluorescence tests 2 h post-infection, antiserum raised against plasma membranes from 24 h-infected cells displayed discrete membrane fluorescence. This antiserum also prevented haemadsorption, while antiserum to uninfected HeLa plasma membranes and anti-whole HeLa cell antisera also inhibited the red cell binding

by up to 50 per cent, possibly by steric hindrance.

The relationship between vaccinia-induced cell surface changes and the extracellular and intracellular forms of poxviruses is discussed.

OBJECTS OF THE RESEARCH

Poxviruses occur in both an intracellular and an extracellular form. Antiserum to extracellular virus neutralises both intracellular and extracellular forms whereas antiserum to intracellular virus neutralises only intracellular virus. (Extracellular is surrounded by a loose outer envelope possibly derived from the host cell plasma membrane.)

A "late" viral antigen, haemagglutinin, is present both in extracellular virus and isolated plasma membranes and *in situ* at the surface of vaccinia-infected cells. The existence of "early" viral antigens at the cell surface has also been demonstrated.

The objectives of this work were:-

1. To quantitate antigenic changes at the cell surface during vaccinia infection of HeLa cells, including the appearance of "early" and "late" viral antigens.
2. To examine the interaction of haemagglutinin *in situ* with antiviral sera, employing a quantitative assay.
3. To isolate and investigate the composition of plasma membranes from vaccinia-infected HeLa cells, and the properties of antiserum to these.
4. To investigate other surface changes in cells following vaccinia-infection.

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LIST OF ABBREVIATIONS

AIPM	rabbit antiserum to plasma membranes purified from vaccinia-infected HeLa cells
antiVAC	hyperimmune rabbit anti-vaccinia serum or γ-globulin concentrate
ara-C	cytosine arabinoside
AUPM	rabbit antiserum to plasma membranes purified from uninfected HeLa cells
BUdR	bromodeoxyuridine
CAM	chorio-allantoic membrane
CDS	citrate-dextrose saline
CED	chick erythrocyte diluent
CFA	complete Freund's adjuvant
Ci, mCi, μCi	Curie, milli-, and micro-
CIC	complete indicator cells
con A	concanavalin A
c.p.e.	cytopathic effect
c.p.m.	counts per minute
CSFM	calf-serum free medium
DEAE-	diethylaminoethyl-
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
E_x	extinction coefficient at x nanometres
ECV	extracellular virus
EDTA	ethylenediaminetetra-acetic acid
FITC	fluorescein isothiocyanate
γG	γ-globulin fraction of whole serum

g	force of gravity
g, kg, mg, μg, ng	gram, kilo-, milli-, micro-, nano-
h	hour(s)
HA_{50}	50 per cent end point in haemagglutination titration
HAD	haemadsorption
HADI	haemadsorption-inhibition
$HADI_{50}$	50 per cent end point in haemadsorption-inhibition assay
HAI	haemagglutination-inhibition
HAI_{50}	50 per cent end point in haemagglutination-inhibition assay
HD_{50}	dilution of complement giving 50 per cent lysis with the optimal sensitizing dose of haemolysin
HEL	human embryonic lung cells
HeSA	soluble antigen preparation from HeLa cells
HVSA	soluble antigen preparation from vaccinia-infected HeLa cells
ICV	intracellular virus
ID	intra-dermal
IM	intra-muscular
INF/UNIN	infected/uninfected
IV	intra-venous
Kc	Kilocycles
l, ml, μl	litres, milli-, and micro-
LCM virus	lymphocytic choriomeningitis virus
LS	"labile-stable" antigen
m, cm, mm, μm, nm	metres, centi-, milli-, micro-, nano-
MEM	minimal essential medium
min	minute(s)
m.o.i.	multiplicity of infection

NγG	normal rabbit gamma-globulin fraction
NADH	nicotinamide adenine dinucleotide
$Na^{+}K^{+}$-ATPase	sodium-potassium-dependent adenosine triphosphatase
NP	"nucleoprotein" antigen
O.D.	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
p.f.u.	plaque-forming unit(s)
p.i.	post-infection
PM	plasma membrane
QHAD	quantitative haemadsorption
Rh	Rhesus
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
RVSA	soluble antigen preparation from vaccinia-infected rabbit dermis
s	second(s)
SARγG	sheep anti-rabbit gamma globulin fraction
SDS	sodium dodecyl sulphate
T cell/lymphocyte	thymus-derived lymphocyte
TCA	trichloroacetic acid
UV	ultra-violet
VBS	veronal-buffered saline
(V)HA	(vaccinia) haemagglutinin
VICSA	virus-induced cell surface antigen
WHO	World Health Organisation

INTRODUCTION

The Poxviridae

Classification, structural features and chemical composition

The family Poxviridae contains the largest and most complex of all viruses. The members are responsible for a wide range of diseases in man and animals including smallpox, molluscum contagiosum, cowpox, monkeypox and mousepox, all of which are characterised by the formation of papules, vesicles and pustules in the skin. Most poxviruses are closely related antigenically, and characteristically replicate and mature in the host cell cytoplasm, whereas most other viruses have their replication sites in the nucleus (Fenner et al, 1974).

Genus A (Table 1) comprises the most intensively studied members, including the type species, vaccinia virus. The virions are brick shaped, containing an ovoid biconcave core or nucleoid where genetic material, cross-linked double stranded DNA (Holowczak, 1976), is located, which may be 122 to 161 x 10^6 daltons (Geshelin and Berns, 1974; Grange, 1974; Esteban, Flores and Holowczak, 1977a). The DNA is surrounded by lipids and proteins of viral origin representing the core, and is enclosed by a protein coat and a layer of regularly arranged coarse fibrils including the two oval-shaped lateral bodies, all composed of protein units (Joklik, 1968). The whole structure is bounded by an outer virion double membrane envelope (Dales, 1963). The type species, vaccinia virus, and also rabbitpox virus, may derive a loose membrane from the host cell (Morgan et al, 1954; Nagington and Horne, 1962; Turner and Squires, 1971; Appleyard, Hapel and Boulter, 1971). The fatty acid and phospholipid complexes existing

Table 1

Family Poxviridae

Genera

A. Orthopoxvirus	B. Parapoxvirus	C. Capripoxvirus	D. Avipoxvirus	E. Leporipoxvirus	F. Entomopoxvirus	Unallocated
Vaccinia	Orf	Sheep-pox	Fowlpox	Myxoma	Entomopoxvirus	Swinepox
Cowpox	Bovine papular stomatitis	Goatpox	Canarypox	Rabbit fibroma		Molluscum contagiosum
Monkeypox		Lumpy skin disease	Pigeonpox	Squirrel fibroma		Yaba monkey tumour virus
Ectromelia	Pseudocowpox		Turkeypox	Hare fibroma		
Variola major	Chamois contagious ecthyma					Tana virus
Alastrim (Variola minor)						
Camelpox						
Buffalopox						

according to Fenner (1976).

within the virion in the form of _internal_ membranes (Dales and Mosbach, 1968) are distinctly different in composition from the host cell membranes, and this may be due to a virus-mediated selection mechanism leading to _de novo_ membrane synthesis (Stern and Dales, 1974). This may not exclude the possibility of host membraneous material being present on the virion after extrusion from the host cell.

Biochemically the virions of genera B and C differ insignificantly from genus A, though anatomically they are somewhat more oval and narrow, while orf virus has a surface structure of spirally arranged tubules (Nagington and Horne, 1962). The Avipoxviruses characteristically have a high lipid content and a large irregular envelope (Todd, Randall and Coniglio, 1958). The virions are similar in structure to those of genus A (Morgan and Wyckoff, 1950). However Müller _et al_ (1977) analysed several poxviruses with restriction endonucleases and showed a high degree of genetic relatedness between individual orthopoxviruses but little genetic relatedness between these and fowlpoxvirus. The viruses of subgenus E are characterised by long growth cycles and the production of tumours in susceptible hosts (Chapple and Westwood, 1963). There are also a number of poxviruses which multiply in insects (Bergoin and Dales, 1971). Several other poxviruses remain to be classified absolutely though most of these have the same basic virion structure. This review will be concerned mainly with the members of subgenus A and in particular, vaccinia virus.

The vaccinia virion has a DNA content of 3 to 5 per cent (Zwartouw, 1964; Joklik, 1966), 4 to 6 per cent lipid (Joklik, 1966)

and 90 per cent protein (Joklik, 1966). The presence of trace amounts of high molecular weight RNA has also been demonstrated within the core (Roening and Holowczak, 1974; Paoletti, 1977). Any carbohydrate present can be accounted for by deoxyribose (Zwartouw, 1964).

Although the poxvirus genome is theoretically capable of coding for approximately 200 proteins (Joklik, 1974), it is extremely doubtful whether this capability is realised. Some thirty to forty polypeptides have been identified within the vaccinia virion (Sarov and Joklik, 1972; Obijeski et al, 1973; MacRae and Szilagyi, 1975; Esposito, Obijeski and Nakano, 1977a), though more may be present (Stern and Dales, 1976) since current techniques cannot detect polypeptides comprising $\leq$ 0.1 per cent of the virion, equivalent to 100 protein molecules per particle (Joklik, 1974).

Poxvirions themselves possess various enzyme activities mainly located within the core. In addition, poxvirus replication may induce the formation of non virion-associated enzyme activities. The enzymes associated with poxviruses are listed in Table 2.

The growth cycle

Some doubt exists as to the nature of attachment and entry of vaccinia virus into the host cell. Electron microscopical studies by Dales and co-workers, reviewed in Dales (1973), implicated viropexis as the prime mode of entry, but later work by Armstrong, Metz and Young (1973) and Chang and Metz (1976) suggested that entry was achieved by an initial fusion between the host cell plasma membrane and the envelope of invading virus particles. Following fusion and pene-

Table 2 Enzyme activities of poxviruses

A Virion-associated

(i)	DNA-dependent RNA polymerase I	Kates and McAuslan, 1967b
(ii)	Nucleotide triphosphate phosphohydrolases I & II	Gold and Dales, 1968; Paoletti and Moss, 1972b
(iii)	Deoxyribonucleases (exo- and endo-)	Pogo and Dales, 1969, 1971
(iv)	Polyadenylate (A) polymerase	Moss, Rosenblum and Paoletti, 1973; Moss, Rosenblum and Gershowitz, 1975; Brown, Dorson and Bollum, 1973; Brakel and Kates, 1974
(v)	Protein kinase	Paoletti and Moss, 1972a; Kleiman and Moss, 1973
(vi)	RNA-methylase	Wei and Moss, 1974
(vii)	Polynucleotide 5'-triphosphatase	Tutas and Paoletti, 1977
(viii)	mRNA guanylyl transferase	Martin, Paoletti and Moss, 1975
(ix)	mRNA (guanine 7') methyltransferase	Martin, Paoletti and Moss, 1975
(x)	mRNA (nucleoside 2') methyltransferase	Martin, Paoletti and Moss, 1975
(xi)	DNA "nicking-closing" enzyme	Bauer *et al*, 1977

B Virus-induced

(i)	Thymidine kinase	McAuslan, 1963
(ii)	DNA-dependent DNA-polymerase	Jungwirth and Joklik, 1965
(iii)	Deoxyribonucleases	Jungwirth and Joklik, 1965
(iv)	dAT-primed poly AU-synthetase	Pitkanen *et al*, 1968
(v)	Polynucleotide ligase	Sambrook and Shatkin, 1969
(vi)	Ornithine decarboxylase	Hodgson and Williamson, 1975
(vii)	DNA-dependent RNA polymerase	Nevins and Joklik, 1977

tration, the outer viral coat is probably deposited and dispersed over the cell surface after degradation by existing host cell enzymes (Chang and Metz, 1976). This leaves the protein-bound DNA-containing core exposed but amenable to transcription. Viral DNA may alter on uncoating from double to single-stranded or "nicked", and cross-links may be removed (Pogo and O'Shea, 1978).

Synthesis of "early" mRNA in poxvirus infected cells occurs in the absence of prior viral DNA replication (Oda and Joklik, 1967; Kates and McAuslan, 1967a) and approximately 14 per cent of the viral genome is transcribed at this stage (Kates and Beeson, 1970). It has been reported that virus-specified polypeptides could be detected at twenty minutes post infection (p.i.), including one which regulates "early" mRNA synthesis (Metz and Esteban, 1972; Esteban and Metz, 1973). Two classes of "early" proteins exist; "early-early" proteins are translated from mRNA transcribed from the viral core before stage II uncoating, while "early-late" proteins originate from mRNA transcribed from "naked" parental DNA (McAuslan, 1969). On the removal of the core coat, which seems to be under virus control (Subak-Sharpe and Pringle, 1975), the viral DNA is rendered susceptible to exogenous DNases (stage II uncoating).

Host cell functions are promptly reduced to a low level by the invading virus (Moss, 1968), and disintegration of cellular DNA begins within 90 minutes of infection (Parkhurst, Peterson and Heidelberger, 1973). Viral DNA replication begins 1.5 to 2.0 hours p.i. at selected sites or "factories" in the host cell cytoplasm (Cairns, 1960), and is complete by 6 hours p.i. coincident with the

appearance of infectious virus (Joklik and Becker, 1964). Viral DNA replication is apparently discontinuous, involving single-stranded DNA intermediates (Esteban and Holowczak, 1977) and replicating DNA contains loops of double-stranded DNA at the ends of the molecule, progressively increasing in size during replication (Esteban, Flores and Holowczak, 1977b). Although evidence has been presented for some degree of nuclear viral DNA synthesis during vaccinia infection (La Colla and Weissbach, 1975), enucleate cells support some viral DNA synthesis but produce greatly decreased quantities of infectious virus (Prescott, Kates and Kirkpatrick, 1971; Pennington and Follett, 1974). It may be that there is a requirement for nuclear processing or maturation of viral DNA.

Many enzymes, both virion-associated or induced by virus infection, have been identified in poxvirus-infected cells and may have a direct involvement in viral DNA, mRNA and protein synthesis (Table 2). Alternatively their function may be degradative or modificational.

As viral DNA is replicated, viral mRNA synthesis switches from "early-late" to "late-early" transcription, despite the continued synthesis of some "early" mRNA. The consequent translation of these mRNA species is concerned principally with the synthesis of virus structural proteins. The use of polyacrylamide gel electrophoresis (P A G E) has been instrumental in elucidating both the number and the temporal appearance of virus-specified polypeptides.

Twenty polypeptides have been detected by 20 min p.i. (Esteban and Metz, 1973) while Pennington (1974) divided a total of 80 detectable

polypeptides into pre- (30 polypeptides) and post- (50 polypeptides) replicative classes. Glycosylation of a small portion of these polypeptides is apparent; Moss, Rosenblum and Garon (1971) detected several "early" virus-induced glycoproteins, while a virion glycoprotein is also present (Moss, Rosenblum and Garon, 1973); the identification of three virus-specified glycopolypeptides in the vaccinia haemagglutinin (VHA) complex is also significant (Ichihashi, 1977).

The process of maturation from naked replicated DNA to fully mature progeny virus particles has been followed by electron microscopy (Morgan _et al_, 1954) and by combined PAGE and electron microscopy (Sarov and Joklik, 1973). The morphogenesis of vaccinia virus has recently been described in detail by Morgan (1976a,b) although an alternative maturation sequence has also been proposed (Stern, Pogo and Dales, 1977).

Poxvirus Antigens and Polypeptides

Poxvirus infection of susceptible host cells results in the production of new viral proteins which can be identified biochemically and immunologically (Wilcox and Cohen, 1969). A proportion of these proteins is incorporated into mature virions, either as structural or enzymic components; some proteins may be induced for purely control purposes and are only present in infected cells, while other proteins may be combinations of the above classes and have dual functions.

Early serological identification

Early work, reviewed by Smadel and Hoagland (1942), identified

two groups of antigens, nucleoprotein antigen (NP) (Craigie and Wishart, 1934) and labile-stable antigen (LS) (Shedlovsky and Smadel, 1942). The NP antigen, an alkaline extract of purified virus, comprised 50 per cent of the virus mass and all of the DNA (Smadel, Rivers and Hoagland, 1942). Woodroofe and Fenner (1962) demonstrated that the "antigen" was comprised of at least two distinctly different antigens, while Zwartouw, Westwood and Harris (1965) demonstrated several precipitin lines on reaction with convalescent anti-vaccinia serum.

Craigie and Wishart (1936) extracted acid-precipitable material (with two serological specificities) from vaccinia virus-infected cells, one of these was heat labile (L) and the other heat stable (S). Antiserum to each of these individual components precipitated both antigenic fractions and thus the LS complex was believed to carry both determinants.

The emergence of more powerful immunological and biochemical techniques has revealed that the LS and NP antigens are complex mixtures of viral components (Westwood _et al_, 1965; Cohen and Wilcox, 1966, 1968), and consequently little further work has been carried out in the last decade into their nature. Any benefit to be derived from continued study of NP antigen is now in doubt.

Viral antigens and polypeptides in infected cells

The rapid cessation of host cell protein synthesis effected by vaccinia soon after infection (Moss, 1968) and its reduction to a very low background level, has allowed analysis of viral proteins in infected

cell extracts to be undertaken with little or no interference from the host cell. Immunodiffusion and immunoelectrophoresis, detecting only the soluble antigens of poxvirus-infected cells, have revealed the presence of only 7 to 20 components (reviewed by Wilcox and Cohen, 1969), significantly less than the 200 proteins for which poxviruses are potentially capable of coding (Joklik, 1974). Appleyard and Westwood (1964a) and Salzman and Sebring (1967) observed virus-specific precipitin lines as early as 1.5 hours p.i, while up to twenty lines could be shown by the end of the virus multiplication cycle.

The virion antigens and polypeptides

The early work referred to (page 8), was performed using crude preparations of virus, grossly contaminated by host material and soluble virus products. Definitive analysis of the proteins of the vaccinia virion could only be undertaken with purified virus (Joklik, 1962a,b). Marquardt, Holm and Lycke (1965) reported that eight of nine soluble antigens detectable by gel diffusion were virus structural proteins, while Westwood et al (1965) found only seven antigens from a detectable total of seventeen, which could be identified in degraded virus particles. Zwartouw et al (1965), using alkaline extracts of purified virus which solubilised about 20 per cent of the nitrogen present, found eight precipitin lines. However, Joklik (1966) argued that much improved solubilisation could be obtained using sodium dodecyl sulphate (SDS), facilitating detection of more viral antigens.

Recent examples of immunological analysis of the vaccinia virion have been concerned with serological comparisons between members of the Poxviridae (Baxby, 1975; Esposito, Obijeski and Nakano, 1977b).

<u>The search for a protective antigen and the existence of intracellular and extracellular forms of poxvirus</u>

The identification of the "protective antigen(s)" of poxviruses would eliminate the need for a "live" vaccine with associated side effects. In the case of the poxviruses, the main aim has been the production of an efficient inactivating procedure, and this has yielded an array of perplexing results (Turner, Squires and Murray, 1970).

The soluble virus antigens have also been studied for protective qualities (Appleyard, 1961), but the most effective antibody response results from infection with live vaccinia virus (Appleyard, Zwartouw and Westwood, 1964). However, in rabbits, inactivated vaccinia can elicit neutralising antibody. This is the case for virus inactivated by formalin (Parker and Rivers, 1936; Amies, 1961; Ramanarao, 1962), alcohol (McClean, 1945), ultra-violet radiation (Collier, McClean and Vallett, 1955; Kaplan, 1962), gamma radiation (Kaplan, 1960) and heat (Madeley, 1968). In most cases, rabbits developed <u>some</u> resistance to challenge with vaccinia virus. Virus multiplication is therefore not essential for the production of neutralising antibody but resistance achieved through an active infection provides the best protection.

It was previously assumed that the degree of protection conferred by a particular inactivated vaccine could be correlated with the level of neutralising antibody induced by it in the animal model and consequently the efficacy of vaccines was assessed in these terms. The findings of Parker and Rivers (1936), Beunders (1964), Kaplan, McClean and Vallett (1962), Ramanarao (1962) and Boulter, Zwartouw and

Titmuss (1964), showed no correlation with this concept. Despite finding high levels of neutralising antibody to inactivated virus on subsequent intradermal (I D) challenge, the degree of protection was low. Boulter (1969) acknowledged the important role played by the cell-mediated immune system and, in spite of the apparent lack of protection even when neutralising antibody levels were high, postulated that the humoral immune system was equally important. Based on the evidence that the passive transfer of serum from rabbits recovered from rabbitpox could prevent death from that disease (Boulter, Westwood and Maber, 1961), and that virus preparations giving rise to high neutralising titres but low protection had been inactivated before injection, Boulter suggested that there was a fundamental antigenic difference between live naturally infectious virus, and virus inactivated by heat, UV, formalin, etc., which, in turn, gave rise to different antibodies. Thus antibodies to inactivated virus might not react with live virus and despite high antibody titres to inactivated virus, little protection would be offered against live virus.

During poxvirus replication, only 10 per cent of virus was released naturally (Smith and Sharp, 1960) and distant spread of infection might be a function of naturally released virus, which would be considerably more important in interactions with the host immune system than cell-associated virus. Electron microscopy of poxvirus particles revealed the occasional presence of an extra outer envelope (Morgan _et al_, 1954; Nagington and Horne, 1962). Appleyard _et al_ (1971) presented evidence that naturally released or extracellular

virus (ECV) differed basically from cell-associated or intracellular virus (ICV) by the acquisition of an outer envelope. However, despite neutralisation results similar to Appleyard et al for each type of virus, Turner and Squires (1971) could find no evidence for the existence of an outer envelope.

The importance of intracellular and extracellular poxviruses and their antisera in immunity, have been reviewed by Boulter et al (1971) and Boulter and Appleyard (1973) and the lack of protection afforded by inactivated vaccines can now be assessed in terms of these observations. The source of virus used for preparation of inactivated vaccines was almost invariably ICV; after inactivation and immunisation, the antisera showed high neutralising titres against the test virus (again, ICV) but failed to protect against subsequent I D challenge with live virus containing ECV and ICV. The properties of ECV and ICV are summarised in Table 3.

Ideally, therefore, the vaccine which should offer maximum protection would contain inactivated ECV. However, inactivation of ECV may result in the destruction or loss of some important antigens, since the antiserum raised was incapable of neutralising either ECV or ICV (Turner and Squires, 1971). ECV may be susceptible to envelope damage since a constant proportion seems to be neutralisable by antiserum to inactivated virus (Appleyard et al, 1971; Baxby, 1972). In addition, ECV, for reasons unknown, seems to be very poorly antigenic (Turner and Squires, 1971; Boulter and Appleyard, 1973), although antiserum did protect against ID challenge (Turner and Squires, 1971).

Table 3 Properties of extracellular and intracellular poxviruses

	Extracellular	Intracellular	Reference
1.	Extra outer envelope	No envelope	Appleyard *et al*. (1971)
2.	Neutralised only by antiserum to live virus (containing both anti ICV and anti ECV antibodies)	Neutralised by antiserum to live and inactivated virus	Turner and Squires (1971); Boulter and Appleyard (1973)
	∴ Both neutralised by different antibodies		
3.	Antiserum prevents distant spread of virus in tissue culture ("comets" not formed)	No prevention by antiserum of "comet" formation	Appleyard *et al*. (1971)
4.	Density in caesium chloride (rabbitpox) 1.23 - 1.24 gml^{-1}	Density in caesium chloride (rabbitpox) 1.27 - 1.28 gml^{-1}	Boulter and Appleyard (1973)
5.	Possibly derives viral antigens from infected cell surface and incorporates in coat	Cell bound	Appleyard *et al*. (1971)
6.	No host cell antigenic components in envelope	-	Turner and Runkel (unpublished) Appleyard and Hapel (unpublished)
7.	Haemagglutinin present on surface	No surface haemagglutinin	Boulter and Thornton (unpublished Payne and Norrby (1976)
8.	Poorly antigenic	Excellent antigen	Turner and Squires (1971)
9.	Antiserum to ECV most important in natural infection and in protection against intradermal challenge	Antiserum to ICV of less importance naturally and despite high neutralising titre does not protect against intradermal challenge to any great extent	Boulter and Appleyard (1973)
10.	First antibody to appear	Appears after anti-ECV	Hutt (1975a)

The very low yield of ECV, its instability and poor antigenicity makes the large scale production of vaccine impracticable. Equally impracticable, is the isolation of protective antigen(s) from ECV. However, by 24 hours p.i., rabbitpox-infected HeLa cells acquire a surface antigen which reacts with antiserum to live virus but not antiserum to inactivated ICV (Appleyard et al, 1971) suggesting that ECV derives its coat from the plasma membrane of the infected cell. The surface membrane of infected cells might therefore be the possible location of a protective antigen although the apparent absence of host components on ECV has still to be explained (Appleyard and Hapel, unpub.; Turner and Runkel, unpub. cited by Boulter and Appleyard, 1973).

Appleyard et al (1964) described a "serum-blocking" antigen in extracts of rabbitpox or vaccinia-infected tissues which reacted with neutralising antibody and induced its formation on immunisation (Appleyard and Westwood, 1964a). In addition, in the case of vaccinia, protection against I D challenge was almost as good as that produced by vaccination with live virus but less effective protection was found with rabbitpox virus soluble antigen preparations. More recent studies have identified a virion component which elicited neutralising antibody to ICV and corresponded to a virion structural surface tubule polypeptide of molecular weight 58,000 (Stern and Dales, 1976).

Cell-mediated Immunity to Poxviruses

Any discussion of immunity to poxviruses must include a brief reference to the interactions of virus-infected cells and free virus with cells of the immune system. Recent experiments by Blanden and

co-workers (reviews, Blanden, 1974; Doherty, Blanden and Zinkernagel, 1976) have demonstrated the important role of circulating T lymphocytes in destroying virus-infected cells by monitoring cell surface alterations occurring as a result of infection. These are discussed later.

Preparations of poxvirus-infected material have been known to confer immunological protection since the time of Jenner (1798) and more recent reports have confirmed this protection, in the form of delayed type hypersensitivity as a T cell-mediated response to poxvirus infection *in vivo* (Lawrence and Valentine, 1970) and *in vitro* (Koszinowski, Kruse and Thomssen, 1975). Using ectromelia virus infection of mice as a model system, the initial response seems to involve the recognition of viral antigen by T cells (Blanden, 1974). This provides an inflammatory response by polymorphonuclear leucocytes and macrophages. Macrophages are the dominant cells in recovery from infection by ingestion and destruction of virus.

Poxvirus and poxvirus antigens are capable of specifically transforming lymphocytes from immune donors (Rosenberg, Farber and Notkins, 1972; Hutt, 1975b). Poxviruses have also been shown to replicate within non-immune polymorphonuclear leucocytes *in vitro* (Benda, Cinate and Plaisner, 1975) and numerous reports have appeared of replication within non-immune but not immune macrophages (Tompkins, Zarling and Rawls, 1970b; Avila, Schultz and Tompkins, 1972; Greer, Delfs and McElree, 1974).

Modifications of the host cell surface induced during poxvirus infection

Specific alterations of host cell surface architecture and chemistry by the insertion or removal of viral proteins, virus-induced lipid exchange and modification or replacement of existing host components by virus-specified proteins, have been recognised during the infection cycles of a number of viruses (Rifkin and Quigley, 1974). Virus induced cell surface changes either by antigenic modifications (Table 4) or by an altered pattern of binding of the lectin, concanavalin A (con A) (Klenk, Becht and Rott, 1974), have been shown to occur during infection by many viruses.

Early antigenic alterations in the poxvirus-infected cell membrane

In terms of the host immune response, early poxvirus-induced cell surface antigens (VICSA) may be important in eliciting antibodies involved in control of, or prevention of widespread infection. In concert with complement, early destruction of virus-infected cells by anti-VICSA may be possible. Later in infection, these antibodies may also be of some importance in allowing entry of neutralising anti-virion antibody to infected cells.

Fagraeus and Espmark (1961) first demonstrated the presence of viral antigens on the surface of vaccinia-infected cells by immune (mixed) haemadsorption. Similar areas of vaccinia-specific antigen on the cell periphery were observed by Morgan, Rifkind and Rose (1962) by electron microscopy using ferritin-labelled antibody. In these early studies, the exact nature of the antigen(s) under study was not

Table 4 Cell surface antigens/proteins induced during productive virus infection

Genus	Virus	Technique	Reference
ALPHAVIRUS	Venezuelan Equine encephalomyelitis	Immunofluorescence	Hahon, 1970
	chikungunya	Immunofluorescence	Mantani and Igarashi, 1971
ARENAVIRUS	Lymphocytic choriomeningitis	Immunofluorescence	Rutter and Gshwender, 1973
FLAVIVIRUS	Dengue	Immunoperoxidase Immune cytolysis	Catanzaro *et al*, 1974
INFLUENZA-VIRUS	Influenza type A	Immunofluorescence	Hahon and Eckert, 1972
PARAMYXO-VIRUS	Mumps	Electron micro-scopical techniques	Mannweiler and Rutter, 1973
LYSSAVIRUS	Rabies	Immune cytolysis	Wiktor, Kuwert and Koprowski, 1968
POLYOMA-VIRUS	Polyoma	Immunofluorescence	Irlin, 1967
HERPES-VIRUS	I & II	Immune cytolysis	Brier *et al*, 1971
	"	Immunofluorescence	Ito and Barron, 1972a
	Varicella-zoster	Immunofluorescence	Ito and Barron, 1973
	Epstein-Barr	Immunofluorescence	Pearson *et al*, 1970
	Marek's disease	Immunofluorescence	Chen and Purchase, 1970
	Cytomegalovirus	Immunofluorescence	The' and Langenhausen, 1972
ORTHO-POXVIRUS	Vaccinia	Immunofluorescence	Ueda *et al*, 1969
	Cowpox	Immune haemad-sorption	Miyamoto and Kato, 1968
	Ectromelia	Immunofluorescence	Gardner, Bowern and Blanden, 1974a
LEPORI-POXVIRUS	Fibroma	Immune haemad-sorption	Miyamoto and Kato, 1968

clarified. Later studies (Miyamoto and Kato, 1968; Ueda et al, 1969) identified early poxvirus antigen(s) located on the cell surface which appeared before viral DNA replication or when this was blocked. Miyamoto and Kato (1968) identified VICSA on two cell lines infected with either Shope fibroma or cowpox virus using immune haemadsorption. In the same test, antiserum to cowpox virus reacted with cells infected with other cowpox strains, two vaccinia strains, ectromelia and variola viruses while a weaker reaction was found with cells infected with Shope fibroma virus. Fluorescent antibody studies confirmed the above results and the authors also established that the immune haemadsorption reaction was not attributable to viral haemagglutinin since no binding of susceptible chicken erythrocytes occurred if cytosine arabinoside (ara-C) was present during infection.

Ueda et al (1969), using antiserum raised to purified vaccinia virus, demonstrated VICSA by immunofluorescence on the membrane of unfixed vaccinia-infected HeLa cells. The antigen was detectable within two hours p.i. and did not require viral DNA synthesis for its expression. At this stage cytoplasmic viral antigens were absent. By use of a mutant which failed to replicate in HeLa cells, but which did produce early VICSA, protein synthesis was shown to be required for expression. Miyamoto and Kato (1971) confirmed this requirement for protein synthesis and suggested that VICSA mRNA was synthesised within one hour p.i.

Ueda et al (1972) exploited the non-replicating dermovaccinia mutant to produce a concentrated preparation of early virus soluble antigens, including VICSA. Antiserum raised in rabbits to this

preparation or to whole mutant-infected rabbit kidney cells reacted specifically with VICSA on HeLa cells by immunofluorescence but not with cytoplasmic viral antigens, had a low neutralising titre and was capable of fixing complement. It was also shown that antiserum to UV-inactivated virus did not react with surface antigen but only cytoplasmic viral antigen. The antiserum prepared by Ueda et al was further tested by Ito and Barron (1972b), who confirmed the lack of vaccinia neutralising activity, noted the lack of haemagglutination-inhibiting (HAI) antibodies and found an immune haemadsorption titre of 1/300,000.

Tanaka and Hatano (1973) made a comparative study of VICSA production in a variety of cell lines infected with cowpox virus and noted that some lines were more efficient VICSA producers, as indicated by fluorescent antibody testing, when viral DNA synthesis was suppressed. Addition of trypsin (0.5 μg ml^{-1}) during the virus adsorption period resulted in a significant increase in VICSA formation although the appearance of cytoplasmic viral antigen was not enhanced. Pretreatment of cells or virus with trypsin led to a similar enhancement. The authors suggested that some cell lines possessed inefficient stage I virus-uncoating systems and trypsin pretreatment of virus or cells facilitated this. This would then enable transcription and translation of early mRNA directed by the virus polymerase, leading to the expression of early functions. Further studies on the expression of cowpox VICSA in VERO cells carrying a strain of Sendai virus, indicated that VICSA was enhanced, possibly because of increased proteolytic enzyme activity in the carrier cells (Tanaka, Morita and Hatano, 1976; Tanaka, Ogura and Hatano, 1977).

Tompkins _et al_ (1970a) used immunofluorescence to detect a cell surface antigen produced during infection of RK cells by fibroma virus, which did not cross-react with anti-vaccinia serum. However, the kinetics of appearance of this VICSA differed from that found with other non-oncogenic poxviruses in that ara-C was inhibitory and the antigen did not appear prior to viral DNA replication. The reaction of immune antiviral serum with fibroma tumours _in vitro_ was found to be weaker than with virus-infected tissue culture cells (Tompkins and Schultz, 1972). This concurred with _in vivo_ findings, where tumours failed to regress in the presence of high levels of cytotoxic antibodies. Earlier studies (Tompkins _et al_, 1970b) indicated the probable involvement of the cellular immune system in response to fibroma virus-induced antigens appearing at the tumour cell surface.

Interactions of the cell-mediated immune system with poxvirus-induced cell surface antigen

Ueda and Tagaya (1973) first suggested an involvement of the cellular immune system relating to the appearance of early poxvirus VICSA. A concentrated early soluble antigen preparation was capable of inducing skin resistance to vaccinia, while neutralising activity against both ECV and ICV was absent; immunisation of rabbits with the early soluble antigen preparation in the absence of adjuvant _enhanced_ ID virus multiplication inducing gross skin lesions. The occurrence of early poxvirus antigens on the cell surface has acquired further importance following the elucidation of the role of cytotoxic thymus-derived (T) lymphocytes in immune surveillance of

virus-infected cells both *in vitro* and *in vivo* (Doherty *et al*, 1976). Virus-immune cytotoxic T cells inhibited growth of vaccinia virus in target cells *in vitro* by destroying them before virus assembly (Zinkernagel and Althage, 1977).

The mouse-derived L-929 cell system has been intensively studied with regard to the appearance of virus-specific antigens at the infected cell surface, the modification of the H-2 transplantation antigens on these cells as a result of virus infection and *in vivo* appearance of specific cytotoxic "killer" T cells directed specifically against target cell surface viral antigens.

Viruses producing VICSA and giving rise to cytotoxic "killer" T cells include lymphocytic choriomeningitis virus (LCM) (Doherty, Zinkernagel and Ramshaw, 1974), ectromelia virus (Gardner, Bowern and Blanden, 1974b), parainfluenza (Sendai) virus (Ertl and Koszinowski, 1976a; Doherty and Zinkernagel, 1976), SV40 (Trinchieri and Knowles, quoted in Doherty *et al*, 1976), measles (Labowskie *et al*, 1974), coxsackie B3 (Wong, Woodruff and Woodruff, 1977) and vaccinia (Koszinowski and Ertl, 1975a; Zinkernagel, 1976). The original finding that immune T cells in mice became sensitised to altered "self" antigens following infection with LCM virus (Zinkernagel and Doherty, 1974a,b) was followed by similar observations with the pox-viruses, ectromelia (Gardner, Bowern and Blanden, 1975) and vaccinia (Koszinowski and Ertl, 1975a). The "killer" T cells so induced lysed specifically only syngeneic target cells compatible at either the H-2K or H-2D genetic locus and infected with the same virus, as measured by the release of ^{51}Cr (Blanden *et al*, 1975; Koszinowski and Ertl, 1975a,b).

Ectromelia-immune spleen cells were shown to specifically lyse target virus-infected L929 cells in the absence of complement by 4 hours p.i. (Gardner et al, 1974a). The effector cells were identified as T cells and no blocking of cytotoxicity by immune or hyperimmune antiviral serum pretreatment of target cells was evident. Ada et al (1976) demonstrated that such virus directed cell surface changes occurred in the absence of viral DNA synthesis. Protein synthesis was required for the development of susceptibility to T cell-mediated cytolysis, and this occurred within one hour p.i. Jackson, Ada and Tha Hla (1976) compared the PAGE polypeptide profiles of purified plasma membranes from target uninfected and 2 to 5 hour infected cells; no major demonstrable difference in plasma membrane protein composition was found at this stage. It was suggested that only minor antigenic changes, not readily detectable as new polypeptides, occurred on the target cell surface, which were however sufficient to render the cells susceptible to T cell-mediated cytolysis.

Koszinowski and colleagues have carried out similar studies yielding similar conclusions with immune T cells from vaccinia virus-infected mice (Koszinowski and Ertl, 1975a,b,c, 1976; Koszinowski and Thomssen, 1975). Virus-specific antigens were found by surface immunofluorescence on infected L929 cells by 2 hours p.i..

Koszinowski and Ertl (1975a) further reported that H-2 alloantisera, with no cross reactivity against vaccinia virus could inhibit the lysis of vaccinia-infected target cells. Blocking was achieved only when an antiserum directed against H-2 antigens of the target cells was added. This suggested that the target antigen might

be either a specifically altered H-2 antigen different from virus cell surface antigens, or a combination of non-specifically altered "self" and virus cell surface antigens. A close relationship between H-2 and VICSA was implied by the finding that vaccinia-infected cells had a reduced capacity to absorb H-2 antiserum while virus specific immunofluorescence was reduced on preincubation of the infected cells with the same antiserum. Proof that the "altered self" hypothesis was correct in the case of vaccinia was obtained by Koszinowski and Ertl (1975b,c) by quantifying possible H-2 alterations by immunofluorescence and enzymatic reduction of H-2 antigens which reduced cell-mediated cytotoxicity without affecting anti-vaccinia antibody-mediated cytolysis. Virus-specified cell surface antigens could be removed by enzyme treatment (Ertl and Koszinowski, 1976b) but resynthesis of these antigens was demonstrable within four hours; host H-2 antigens were not, however, resynthesised, presumably because of the general breakdown of host metabolism.

Koszinowski and Ertl (1976) suggested an important role for early vaccinia-coded cell surface antigens in both the generation of cytolytic T cells _in vivo_ and as the target antigens involved in T cell immune cytolysis. Early VICSA production was common to cells infected with each virus strain tested, although the Elstree (Lister) strain was an inefficient producer (see also Ito and Barron, 1972b). Antibody to purified virions however did not react with VICSA, suggesting the existence of distinct non-virion antigens at the vaccinia-infected cell surface.

Demonstration of poxvirus VICSA by radioimmunoassay

The effect of antibody and complement on vaccinia virus-

infected cells *in vitro* has been examined by Brier *et al* (1971) who proposed that several factors influenced cell destruction, notably the density of viral antigens on the cell surface, the nature of the antiviral antibodies and the presence of anti-immunoglobulins. However, a substantial increase in ^{51}Cr release was only found by 10 h p.i. suggesting that either VICSA did not appear until this time or that they were not of a sufficiently high density to be assayed earlier.

Hayashi, Rosenthal and Notkins (1972) measured the binding of ^{125}Iodine labelled rabbit anti-vaccinia γ-globulin to vaccinia-infected cells. By one hour p.i., three to four times more antibody had bound to infected than uninfected monolayers and binding increased precipitously from 5 h. The early binding of antibody may have been a result of the high m.o.i. (multiplicity of infection) employed rather than early viral antigens, resulting in some deposition of virion material on the cell surface but later than 2 hours p.i. new antigens were actively synthesised.

The binding of ^{125}Iodine-labelled rabbit anti-vaccinia γ-globulin was also investigated by Harry and Medzon (1974). Binding to infected monolayers was found to decrease from 0 to 30 minutes while from 45 minutes p.i. to 12 hours p.i., binding increased. The presence of "early" VICSA was suggested by the fact that no DNA synthesis was required for its appearance, although RNA and protein synthesis were essential. The "early" virus induced cell surface antigen was not blocked by antiserum to host cell components.

Poxvirus-induced changes in cell surface structure and composition

A series of papers by Bandlow and others indicated that cells infected with vaccinia virus were more efficient than uninfected cells in eliciting an antibody response to host cell antigens, when injected into guinea-pigs, i.e. the virus had an adjuvant effect (Bandlow, Kieling and Thomssen, 1971). This effect was present prior to virus maturation and was attributable to viral antigens on the cell surface (Bandlow, Fischer and Thomssen, 1972). The adjuvancy seemed to be located in an isolated plasma membrane fraction from vaccinia-infected cells (Bandlow, Koszinowski and Thomssen, 1973). Increases in antibody levels against host cell antigens were apparent and an increase in cell-mediated immunity was also noted (Bandlow and Koszinowski, 1974). Live virus was not required and the appearance of "adjuvancy" seemed to be an "early" event. The adjuvancy was attributed to an intimate association of viral and host antigens.

Bandlow et al (1973) noted that in addition to displaying early viral antigens on the infected cell surface, the cells became agglutinable by con A (2 h p.i.). Early changes in the virus-infected cell surface, resulting in increased con A agglutinability, occur generally on virus infection (Tevethia et al, 1972; Becht, Rott and Klenk, 1972; Salzberg and Raskas, 1972; Ludwig, Becht and Rott, 1974) as well as on viral transformation (Inbar and Sachs, 1969).

Zarling and Tevethia (1971) observed the same effect during vaccinia-infection of rabbit kidney cells as early as 2 hours p.i.. Heat-inactivated or neutralised virus failed to induce agglutinability and the possibility of a virion component being responsible was

excluded, since viral protein synthesis but not DNA synthesis was required. The authors suggested the component responsible for agglutination might be a viral glycoprotein. Since trypsin could induce agglutinability of normal cells by exposing con A binding sites (Burger, 1969), the insertion of a viral glycoprotein at the cell surface might cause rearrangement of host components leading to the same situation.

Bubel and Blackman (1975) assayed con A-binding sites using mouse erythrocytes reacted with con A-treated vaccinia-infected HEp-2 cells. Red cell binding increased from 3 hours p.i. and the binding sites were distinct from the VHA binding sites. Binding sites for con A were identical to those induced in Newcastle disease virus-infected cells. Bubel and Lambert (1967) suggested that increases in auto-agglutinability of cells during vaccinia infection were attributable to the deposition of acidic mucopolysaccharides on the cell surface. Sialic acid synthesis was related temporally to virus appearance and accumulated particularly on the cell surface. However Bubel and Blackman (1975) failed to remove surface sialic acid by neuraminidase treatment. The increase in sialic acid synthesis on vaccinia infection of HEp-2 cells was concluded to result in increased intracellular deposition of sialic acid.

An increase in cell size after vaccinia infection leading to a decrease in buoyant density was demonstrated by Ball and Medzon (1973). This shift was equated with virus-induced cell surface changes and was present by 2 hours p.i. Further studies (Ball and Medzon, 1976) established that the density shift was due to the

synthesis of an "early-early" virus-coded protein and did not require viral DNA synthesis.

Cytopathic effects of poxviruses

Invasion of susceptible cells by poxviruses leads to a readily recognisable early rounding (Brown, Mayyasi and Officer, 1959). A relationship between the cytopathic effect (c.p.e.) and viral protein synthesis was indicated by Appleyard, Westwood and Zwartouw (1962). Viral antigens could be found by 90 minutes p.i. at a stage when cell rounding had begun. Non-replicating virus induced rounding and also the synthesis of some viral antigens, but the toxicity could not be attributed to inoculum virus. Inhibitors of viral protein and RNA synthesis protected infected cells from c.p.e. (Bablanian, 1968). When these inhibitors were removed viral c.p.e. began almost immediately suggesting that a viral protein(s) was responsible for early rounding. Bablanian (1975) reported that early c.p.e. was related to the m.o.i., was actually faster when this was low and seemed to require a background of host protein synthesis. He suggested that virus inhibition of host protein synthesis was not mediated by a virion component, but was associated with synthesis of viral RNA.

Late c.p.e., represented by the fusion of rounded cells into polykaryocytes (Appleyard et al, 1962) follows virus replication. This may be associated with the accumulation of cell surface material discussed earlier (Bubel and Lambert, 1967). Fusion may be exogenous (Mbuy and Bubel, 1976) or endogenous (Kaku and Kamahora, 1964;

Ichihashi and Dales, 1971). Late vaccinia c.p.e. may also be associated with an increase in activity of host lysosomal enzymes as a response to infection (Allison and Sandelin, 1963) although this has been questioned (Wolff and Bubel, 1964).

The toxicity of viral proteins acting intracellularly has been shown by Stephen *et al* (1974); HeLa cells were killed by inducing the uptake of a virus-free soluble antigen fraction from vaccinia-infected HeLa cells. The cytotoxic factor was tentatively identified as a virus-coded protein of molecular weight 30-100,000 daltons (Wolstenholme *et al*, 1977).

Poxvirus haemagglutinin

Introduction. Nagler (1942) found evidence for the existence of a by-product of vaccinia virus infection of chorioallantoic membranes (CAM) which specifically agglutinated approximately 50 per cent of adult fowl erythrocytes. This was in contrast to the large range of red blood cells found by Hirst (1941) to be agglutinable by influenza virus.

Burnet and Stone (1946) demonstrated that vaccinia haemagglutinin (VHA) was separable from infectious virus particles by absorption with susceptible fowl red blood cells. Initial attempts to elute VHA from the agglutinated red cells were unsuccessful, but treatment with anti-vaccinia hyperimmune serum did remove VHA, and the inclusion of antiviral serum in VHA titrations inhibited agglutination.

Haemagglutinin was serologically specific, inactivated at pH 5.0 or by moderate heat, inhibited by Ca^{2+} ions, precipitated by 50 per cent saturated ammonium sulphate, oxygen-labile and was inactivated by <u>Cl. perfringens</u> α-toxin and cobra venom (Burnet, 1946 Burnet and Stone, 1946; Stone, 1946a). There have been no other reports of oxygen lability, and other workers have emphasised the heat stability of VHA (Chu, 1948a; Gurvin and Haukenes, 1976; Tagaya <u>et al</u>, 1977).

<u>Purification and properties</u>. Chu (1948a,b), in an extensive study of the chemical and immunological properties of crude VHA, reported a particle size of 65 nm, a tissue-dependent density of 1.09 to 1.11 gm l^{-1}, and stability between pH 5.92 and 10.79. Virus-specific HA could be recovered by boiling VHA/anti-VHA complexes eluted from agglutinated red cells. Chu concluded that VHA was a virus-host complex unrelated to the neutralising antigen(s) of the virus particle.

Later workers attempted to improve the purity of the VHA used for chemical and immunological analysis. More than one virus specified HA was found by Gillen, Burr and Nagler (1950); McCrea and O'Loughlin (1959) and Youngner and Rubenstein (1959). The latter authors subsequently found evidence for a "soluble" HA in uninfected CAMs (Youngner and Rubenstein, 1962). Lipid analyses of both uninfected and vaccinia-infected CAMs showed that infected membranes contained less phospholipid than uninfected controls while the semi-purified non-viral "soluble" HA had a high triglyceride content with phosphatidyl choline as the major phospholipid (Gausch and Youngner,

1963). It was suggested that VHA was an abnormal lipoprotein of virus-host origin, formed under conditions of increased cell lipid and protein (Gausch and Youngner, 1963). Anthony et al (1970) found three distinct virus-specified HAs in variola virus-infected CAMs: a low density, heat-labile HA, a higher density, heat-labile HA (possibly an aggregate of the above), and a heat-stable intra-virion HA released by reducing agents.

Gurvin and Haukenes (1973) separated VHA from infectious virus particles at a density of 1.08 to 1.18 gm l^{-1}. Material from a second-stage flotation failed to exhibit bands on PAGE or to precipitate with antiviral serum on immunodiffusion. Neff, Ackermann and Preston (1965) found significant variation in the buoyant density of VHA from various tissue cultures and the original buoyant density was retained following recentrifugation. The authors emphasised the heterogeneity of VHA and its susceptibility to aggregation at 4°C in concentrated preparations; the drop in titre was restored by sonication. Gurvin and Haukenes (1976) noted that VHA was inactivated by trypsin treatment, in contrast to the trypsin-resistant HA of Youngner and Rubenstein (1962). No evidence was found for the presence of carbohydrate, and, contrary to previous findings (Burnet and Stone, 1946), the HA activity was resistant to lipase and phospholipase treatment; VHA was heat stable (56°C for 24 hours) and stable between pH 5.0 and 9.0 at 37°C. Gurvin and Haukenes reported a sedimentation coefficient of 50S for VHA.

The separation of VHA into components and subsequent "reconstitution" was described by Smith, Pratt and Baxby (1973).

After the removal of debris from disrupted cowpox or vaccinia virus-infected cells, VHA was deposited by high speed centrifugation, extracted with organic solvents and subjected to column chromatography. Some fractions showed high non-specific HA activity, but did not react with anti-HA, while other fractions blocked anti-HA but did not haemagglutinate. Recombination of fractions restored the compatibility of antibody-blocking and HA activities. The antibody-blocking fractions had a high protein content and no lipid, while the HA rich fractions had a high lipid content. No reconstitution was possible if the corresponding HA fractions from an HA-negative strain of rabbitpox virus were employed or if the corresponding "antibody-blocking" fractions were mixed with HA-positive fractions. This established a specific haemagglutinating and antigenic combination of lipid and protein components only from HA-positive strains. A recent study by Ichihashi (1977) however implied that combination between the poxvirus specific HA protein component and <u>any</u> phospholipid restored HA activity. It was suggested that the insertion of VHA-specific protein into liposomes might be essential for the enhancement of heat stability and the acquisition of antigenicity.

<u>The range of red blood cells agglutinated by VHA</u>. Early studies by Nagler and colleagues (Nagler, 1942, 1944; Clark and Nagler, 1943) established that extracts of vaccinia virus-infected tissue specifically agglutinated red cells from 50 per cent of adult chickens. Embryonic chick red cells were not agglutinated but by six months approximately 50 per cent of these chickens had red cells susceptible to agglutination.

Of a wide range of other red cells tested (Table 5) the only non-fowl species regularly giving agglutination with VHA was the mouse.

Table 5 The agglutinability of red blood cells by poxvirus haemagglutinin

Species	Result	Reference
chicken	±	3
chick embryo	-	2
turkey	+	5
pigeon	±	1, 2
cat	+	4
horse	-	3, 4
human	- (+)	1,(4)
sheep	-	1, 3, 4
rabbit	- (+)	1,(4)
mouse	+	1, 2, 4
rat	- (+)	1,(4)
pig	+	4
cow	-	2, 4

1. Burnet and Stone, 1946
2. McCarthy and Helbert, 1960
3. Nagler, 1942
4. Clark and Nagler, 1943
5. Datt, 1964

Key

+ erythrocytes agglutinated

± weak or doubtful reaction

- erythrocytes not agglutinated

( ) indicates opposing result

Datt (1964) and Joseph (1974) showed that turkey erythrocytes were uniformly agglutinable by VHA; from a total of 214 birds tested by Datt, only one failed to react.

Gilmour and Allison (1969) reported that agglutinability by fowl erythrocytes was genetically determined by the blood group factor Vh, unlinked to groups C, L, and N and probably A and B. This was a dominantly inherited trait, while failure to agglutinate was a recessive autosomal trait. The alleles determining the K system of red blood cell iso-antigens were found to control the susceptibility to agglutination (Brown, Briles and Brown, 1973).

Haemadsorption to poxvirus-infected cells. Tissue culture cells infected with influenza virus were shown to adsorb guinea-pig red blood cells (Vogel and Shelokov, 1957); a reaction specifically inhibited by antiserum to the infecting virus. Other viruses, including vaccinia, were shown to cause similar adsorption of various red blood cell types to the surface of infected cells (Shelokov, Vogel and Chi, 1958). The reaction, termed "haemadsorption," could also be demonstrated with virus-infected tissue sections (O'Connell et al, 1964). Driessen and Greenham (1959) attempting to correlate haemadsorption (HAD) with a property of VHA, found that some fowl cells were HAD positive, while appearing to be negative in the HA test. They therefore postulated an independent factor responsible for HAD. Fujio (1962) and Dekking and van Dillen (1968) however found complete correlation between HAD and HA. In an extensive study of VHA, Blackman and Bubel (1972) correlated the temporal appearance of VHA and HAD at about 9 h p.i.. Calcium ions,

which inhibit poxvirus HA (Burnet and Stone, 1946; Williamson, 1966), inhibited HAD also, as did sucrose, while the binding of red cells was abolished by trypsin. Plasma membrane ghosts from vaccinia-infected cells also adsorbed sensitive red cells, suggesting an intimate association of VHA with the outer cell membrane.

Bubel and Blackman (1975) reported that the appearance of progeny virus preceded HAD by 90 min. The binding site for VHA-susceptible fowl red cells was different from that for con A.

Ichihashi, Matsumoto and Dales (1971) and Ichihashi and Dales (1971), comparing HA^+ and HA^- vaccinia strains, noted that ferritin-labelled antibody bound to the plasma membrane of HA^+ but not HA^--infected cells and suggested that VHA became inserted into the plasma membrane. HA^+ virus, unlike the negative mutant, migrated "naked" to the cell surface and probably associated with the modified plasma membrane just prior to release. VHA was detected in infected cells by 10 h.p.i., rising to a maximum at 18 h.. Cells became HAD^+ by 15 h p.i. and on examination by electron microscopy, the bound red cells were in intimate contact with the cell membrane. Cytoplasmic fluorescence of viral antigen was evident by 7 h p.i. and cell surface fluorescence by 10 h p.i.. Saburi et al (1977) demonstrated by scanning electron microscopy that vaccinia-induced HAD of susceptible red cells occurred either by direct, close binding to the tissue culture cells, by binding through microvilli of infected cells or by binding of projections of the red cells.

Electrophoretic analysis of plasma membranes from vaccinia-

infected HeLa cells by Weintraub and Dales (1974) showed the following results. Pre-existing host plasma membrane proteins and glycoproteins were <u>not</u> modified. A major plasma membrane glycoprotein, synthesised after infection by an HA$^+$ strain, was ~~identified~~ suggested as a component of VHA. This was absent from the plasma membranes of cells infected with HA$^-$ virus although a polypeptide portion of the glycoprotein <u>was</u> synthesised but lacked carbohydrate. The glycoprotein was synthesised and inserted late in infection ($\sim$ 10 h p.i.) and this could be prevented by inhibitors of viral DNA replication but not rifampicin. The carbohydrate moiety included fucose and glucosamine residues, and thus was distinct from the simpler virion glycoprotein (Holowczak, 1970; Garon and Moss, 1971; Sarov and Joklik, 1972). Evidence was found for a possible virus-specified VHA precursor in the plasma membrane. Immunological studies suggested that the insertion of new surface components caused surface antigenic rearrangement. VHA was distributed evenly on the cell surface without capping (c.f. Ichihashi and Dales, 1971).

These findings were extended by Ichihashi (1977) who identified a VHA-specific glycoprotein complex associated with a microsome fraction from cells infected with an HA$^+$ strain of vaccinia. The complex comprised three glycopolypeptides of molecular weights, 150,000, 34,000 and 12,000 daltons. The first, a polymeric form of the second, was equivalent to the major plasma membrane virus-specified glycoprotein of Weintraub and Dales (1974). Synthesis of a <u>host</u> cell membrane protein (24,000 daltons) continued until 6 h p.i. when virus-specific glycosylation of this molecule began. Polymerisation of the VHA-glycosylated host protein was proposed to occur almost immediately within the membrane.

The association of VHA with the virus particle. The question of the existence of a specific HA associated with vaccinia virions has until recently been unresolved because of the difficulties in obtaining highly pure virus free from residual contaminating VHA and in demonstrating it to be so. Burnet and Stone (1946) first showed that reduction of the HA_{50} titre by absorption with susceptible red cells did not reduce the virus titre, and similarly high speed centrifugation of virus did not reduce the HA titre significantly. Thus virus and HA seemed to be separable entities apparently confirmed by other evidence (Ichihashi and Dales, 1971; Blackman and Bubel, 1972). Using highly purified virus, Joklik (1962a,b) and Zwartouw, Westwood and Appleyard (1962) showed that on centrifugation on sucrose density gradients, virions and VHA were readily separable and the virions had no demonstrable HA activity. Westwood et al (1965) found that antiserum raised to gradient purified virus could not inhibit haemagglutination.

However Anthony et al (1970) using variola virus, purified by sucrose density gradient centrifugation and disrupted with SDS, urea and 2-mercaptoethanol, isolated a heat-stable HA free of host antigens. Marquardt (1971) also demonstrated that purified vaccinia virus contained a virus-specified HA, only found when particles were disrupted by pepsin treatment. Immunisation with this material elicited an antibody response to VHA.

Studies by Boulter and Appleyard (1973) on the existence of extracellular and intracellular forms of poxviruses, suggested that the extra envelope derived by ECV might contain cell surface components and in particular VHA. Preliminary observations (Boulter and Thornton,

unpublished) indicated that ECV did indeed have HA activity. Further observations (Payne and Norrby, 1976) showed that ECV from HA^+ strains adsorbed to susceptible red blood cells, whereas ECV (HA^-), ICV (HA^+) and ICV (HA^-) viruses did not. The removal of ECV (HA^+) virus by adsorption led to a concomitant drop in the HA_{50} titre and infectious virus titre of the residue. Adsorption could be inhibited by antibody to HA^+ virus. These observations suggested the presence of VHA in the envelope of ECV which may partially explain conflicting reports on the existence of virion HAs.

Kinetics of VHA appearance

"Cytoplasmic" VHA has been detected approximately 10 h p.i., just after the appearance of mature virus (Baxby and Rondle, 1968; Blackman and Bubel, 1972; Weintraub and Dales, 1974). As a late cytopathic effect after cell rounding, vaccinia-infected cells may fuse to form giant multinucleate cells or polykaryocytes (Appleyard et al, 1962; Kaku and Kamahora, 1964). The latter authors demonstrated that $fusion^+$ strains of vaccinia were VHA^- and vice versa. Inhibitors of viral DNA, RNA and protein synthesis prevented fusion although mitomycin did not. Ichihashi and Dales (1971) confirmed the independence of fusion and HA capacities, and suggested that the fusion-inducing component was carried by mature virus to the cell surface from within host cells. Acquisition of VHA at the cell surface may be linked to inhibition of fusion possibly by surface modification, and cell fusion is by implication a recessive characteristic.

Weintraub and Dales (1974) found a similar polypeptide composition of plasma membranes from cells infected with $fusion^+$ HA^-

and fusion$^-$ HA$^+$ strains. However in the case of the fusion$^{(-)}$ HA$^+$ strain, only one polypeptide component had a carbohydrate residue. This implied that the conversion from fusion $^{(+)}$ to fusion$^{(-)}$ was associated with the acquisition of a carbohydrate residue(s) on the putative VHA glycoprotein. Later, Weintraub, Stern and Dales (1977) showed that addition of 2-deoxy-D-glucose or glucosamine inhibited glycosylation of virus-specified proteins. The blocking of ^{3}H-fucose incorporation was correlated with inhibition of glycosylation, HA activity and virus infectivity but there was no effect on capacity for cell fusion.

Stern and Dales (1976) isolated surface tubules from purified vaccinia particles which were comprised of homogeneous polypeptide of molecular weight 58,000 daltons. This polypeptide which carried both neutralising and cell fusing capacities corresponded to polypeptide 4c of Sarov and Joklik (1972).

Inhibitors of poxvirus HA

The effects of metabolic inhibitors on the production of VHA are summarised in Table 6 and confirm the concept of VHA as a late antigen appearing after virus replication. Rifampicin acts at a late stage in maturation (Follett and Pennington, 1973) arresting the further development of virus, but at a stage after VHA has been synthesised. The role of bromodeoxyuridine (BUdR) is less clear. High doses of BUdR had little effect on VHA synthesis although yields of infectious virus were insignificant with minimal doses of the drug (Baxby and Rondle, 1968; Aasen and Haukenes, 1972). As with rifampicin, immature viral membranes accumulate in the cytoplasm when BUdR

Table 6 Effect of metabolic inhibitors on production of poxvirus haemagglutinin

	Possible area of inhibition	Infectious virus	VHA	Reference
1.	DNA synthesis			
	a) bromodeoxyuridine	-	+	Baxby and Rondle (1968); Aasen and Haukenes (1972
	b) fluorodeoxyuridine	-	-	Loh and Payne (1965); Aasen and Haukenes (1972)
	c) hydroxyurea	-	-	Baxby and Rondle (1968); Aasen and Haukenes (1972)
	d) cytosine arabinoside	-	-	Baxby and Rondle (1968); Aasen and Haukenes (1972)
	e) mitomycin C	-	+	(Oda 1963b)
		-	-	(Aasen and Haukenes (1972)
	proflavine	-	-	Baxby and Rondle (1968)
2.	RNA synthesis			
	a) actinomycin D	-	-	Fujio (1963); Baxby and Rondle (1968); Aasen and Haukenes (1972)
	b) isatin-β-thiosemicarbazone	-	-	Aasen and Haukenes (1972)
3.	Protein synthesis			
	a) cycloheximide	-	-	Aasen and Haukenes (1972)
	b) puromycin	-	-	Ichihashi and Dales (1971)
4.	Maturation			
	a) rifampicin	-	+	Aasen and Haukenes (1972); Weintraub and Dales (1974)
4.	Other			
	a) azide	-	-	Baxby and Rondle (1968)

is present (Easterbrook and Davern, 1963) and synthesis of several virus soluble antigens occurs (Appleyard and Westwood, 1964b). Discrepancies regarding the effects of mitomycin C on VHA synthesis, can possibly be attributed to varying test conditions. Inhibitors, either of VHA synthesis or of the red blood cell-VHA interaction, have been detected in a number of tissues. Burnet and Stone (1946) described the non-specific agglutination of VHA-susceptible red blood cells by tissue extracts and Stone (1946b) demonstrated the affinity of lipid dispersions for the same red cells. Agents such as calcium ions and phospholipases are regarded as inactivators rather than inhibitors of VHA (Stone, 1946a).

Nagler (1942) reported that calf lymph with a high virus content contained little VHA. Virus of similar titre, grown on CAMs was rich in VHA, and Chu (1948b) reported a VHA-inhibitor in calf lymph. Some vaccinia strains were shown to produce VHA in rabbit but not calf dermis (Stone and Burnet, 1946) and Rondle (1969), who fractionated extracts of calf dermis by gel filtration, recovered VHA blocking activity in only one fraction. The inhibitor was thought to be a single protein and was not an immunoglobulin.

Collier (1949) demonstrated a VHA inhibitor from crude extracts of human lung while Szathmary (1960) found inhibitors of VHA in various human body fluids and tissue extracts. These were thermostable and inactivated by trypsin/kaolin.

In the above cases, it is likely that inhibition of VHA occurred by biophysical means rather than by suppression of HA synthesis. However studies by Cassel and colleagues have established that HA^+

strains of vaccinia lose the capacity to produce VHA on prolonged passage in Ehrlich ascites tumour cells; subsequent passage in embryonated eggs and immunisation of rabbits showed that the virus had lost the capacity to synthesise VHA (Cassel, 1957; Cassel and Fater, 1958) but not to replicate (Cassel, Garrett and Blair, 1962). It was suggested that this resulted from exposure to an inhibitor in the ascitic plasma which was a non-dialysable, acid-labile, thermo-labile protein, with an estimated molecular weight of 209,000 (Lau and Cassel, 1972).

Antibody to poxvirus HA and its role *in vivo*. Nagler (1942) noted that haemagglutination could be inhibited by antiviral serum. Antibody to VHA has been used to quantitate VHA by the HAI test (e.g. Joseph, 1974) and HAD of susceptible red cells can also be inhibited by antiviral hyperimmune serum (Driessen and Greenham, 1959). The neutralising and HAI specificities of anti-vaccinia serum were separated by Oda (1963a) who absorbed out neutralising antibodies without affecting the HAI titre. In addition, antiserum to purified virus did not inhibit haemagglutination (Westwood *et al*, 1965) and VHA$^-$ strains did not elicit HAI antibodies (Ichihashi and Dales, 1971). The role of HAI antibodies in protection has been discussed by Lybing and Hedstrom (1975). Their results suggested that the levels of anti-VHA antibodies correlated with the degree of skin immunity elicited by immunisation either with inactivated or live virus preparations. Turner *et al* (1970), however, reported that HAI antibody was a poor indicator of immunity afforded by inactivated vaccines, as was complement fixing antibody. McCarthy, Downie and

Bradley (1958) reported that poxvirus complement fixing and HAI antibodies were not always detectable or even increased after secondary stimulation and Madeley (1968) found that only large doses of inactivated vaccine could induce HAI antibody; indeed, a lack of HAI antibodies raised to an inactivated poxvirus preparation, could be taken as evidence of complete inactivation. Although the role of HAI antibody in immunity is unclear, the facts that the antibody is present in high titre, and that HA is present in large quantities on the surface of poxvirus-infected cells, imply that a dynamic antigen-antibody interaction exists where the infected cell membrane and the immune system are in contact. The elucidation of the importance of early poxvirus VICSA in stimulation of the host cell-mediated immune system (Koszinowski and Ertl, 1976) emphasises the importance of these non-virion induced antigens: VHA stimulation of the host immune system may also be possible, and the resultant immune defences in the form of antibody or lymphocyte may play some part in hindering the spread of infectious virus or the destruction of host cells.

The demonstration of the existence of a "naked" intracellular form and an "enveloped" extracellular form of poxvirus (Appleyard et al, 1971; Turner and Squires, 1971) allowed speculation both on the possible origin of this outer coat, and on the apparently differing susceptibilities of each virus type to neutralisation and the differing properties of antisera to each. ECV differs antigenically from ICV and appears to be important in virus spread. Antibody to ECV neutralises ECV and ICV while anti-ICV is only effective against ICV (Boulter and Appleyard, 1973). Payne and Norrby (1976) demonstrated that one of the "new" antigens acquired by ECV was VHA. This finding

emphasised the possible importance of VHA in poxvirus infection and suggested that ECV derived cell surface VHA as part of its envelope.

A study of poxvirus-induced cell surface antigens (including VHA) might provide some information on the interactions of infected cells with the immune system. Also, changes at the cell surface during poxvirus infection can be detected in terms of con A agglutination (Zarling and Tevethia, 1971) and infected cells may also bind con A to a greater extent (Bubel and Blackman, 1975).

The purpose of this thesis is to examine changes in the surface membrane of vaccinia-infected cells with particular regard to antigenic changes of potential importance in stimulating an effective anti-viral response by the host.

MATERIALS AND METHODS

A. CHEMICALS

L-1-^{3}H-Fucose and Na ^{125}I (carrier-free) were purchased from the Radiochemical Centre, Amersham.

The following chemicals were obtained from Sigma Chemical Co. Ltd. -

Concanavalin A, N-acetyl neuraminic acid, sodium metaperiodate, p-nitrophenyl phosphate (di-tris salt), calcium bis p-nitrophenyl phosphate, phenolphthalein β-glucuronide, β-nicotinamide adenine dinucleotide (reduced, disodium salt), human serum albumin (fraction V), cytochrome c (grade II ex horse heart), lysozyme, actinomycin D, hydroxyurea, cytosine arabinoside, rifampicin.

Trypsin (2x crystallised), soy bean trypsin inhibitor, cycloheximide (actidione), histidine monohydrochloride and chloramine T were obtained from Koch-Light Ltd.

Unless otherwise stated, all other chemicals were supplied by British Drug Houses Ltd., and were of "Analar" grade.

B. IMMUNOLOGICAL REAGENTS

Rabbit anti-sheep red cell serum (haemolysin), goat anti-rabbit precipitating serum and complement (freeze-dried ex-guinea pig) were obtained from Wellcome Laboratories. Sheep anti-rabbit immuno-globulin (fluorescein isothiocyanate-conjugated) was purchased from Gibco-Biocult Ltd. Complete Freund's adjuvant was obtained from Difco Ltd.

C. TISSUE CULTURE REAGENTS

Unless otherwise stated, cells were cultured in Eagle's Minimal Essential Medium with Earle's salts (M.E.M.) ("Autopow", Flow Labs Ltd.). New-born calf serum (mycoplasma free) (Flow) was added to 10 per cent and L-glutamine (Flow) to 2 mM. Crystamycin (Glaxo Labs Ltd.) and/or kanamycin sulphate ("Kannasyn", Winthrop Labs.) were routinely included at concentrations of 50 units ml^{-1} and 100 μg ml^{-1} respectively.

The pH of the medium was adjusted by the addition of 3.0 ml of 7.5 per cent sodium bicarbonate per 500 ml. Serum was omitted during maintenance or virus infection (serum-free medium or SFM).

Trypsin, obtained from Gibco-Biocult Ltd. as a 2.5 per cent solution in salts, was diluted 1/10 in PBS for detachment of monolayer cells.

D. CELL LINES

Cells were cultured routinely in 75 cm^2 and 120 cm^2 glass tissue culture flasks with screw caps, and occasionally in Winchester bottles. All glassware was immersed for at least 24 hours in chloros (I.C.I.), followed by a further 24 hours in 0.5% Divolab (Diversey Ltd.). Finally the glassware was thoroughly washed with tap water before rinsing with either deionised or distilled water, drying and sterilising.

In most experiments, cells were cultured in 3.5 cm vented plastic petri dishes (Flow Labs Ltd.) which were incubated in plastic sandwich boxes, under an atmosphere of 5 per cent carbon dioxide in air.

BSC-1 cells were obtained from Dr. T.H. Pennington, Department of Virology, University of Glasgow and were grown in Eagle's MEM containing 10 per cent foetal bovine serum (Flow).

BHK-21 (C13), HEp-2, HeLa, RK-13, human embryonic lung (HEL), L-132 and L-929 cell lines were obtained from Gibco-Biocult Ltd. BHK-21 (C13) cells were cultured in Eagle's MEM (Glasgow modification) with Earle's Salts (Gibco-Biocult) supplemented with 10 per cent tryptose phosphate broth (Gibco-Biocult). HEL cells were grown in Eagle's MEM containing 10 per cent foetal bovine serum. RK-13 cells were grown in Parker's medium 199 (Gibco-Biocult).

Other cell lines were cultivated in Eagle's MEM containing 10 per cent new-born calf serum.

E. VIRUS

The strain of vaccinia virus described by Westwood *et al* (1966), "Liverpool" strain, derived from the Lister Institute Strain, was used as a crude extract of infected rabbit dermal pulp. This was originally obtained from Dr. E.A. Boulter, Microbiological Research Establishment, Porton Down, Wiltshire. Some stock virus was passaged in HeLa cells and stored as a crude suspension of titre 1 to 10 x 10^7 plaque forming units (p.f.u.) ml^{-1}.

F. RED BLOOD CELLS

Turkey red cells were obtained by courtesy of British United Turkeys Ltd., Fenton Barns, Dirleton, East Lothian. Red cells were collected from the jugular vein of freshly slaughtered turkeys and pooled in 3.8 per cent sterile trisodium citrate.

Other red blood cells were obtained from the following sources:-

human	:	self (group "O")
horse	:	defibrinated (Gibco-Biocult Ltd.)
African Green monkey	:	Flow Labs Ltd.
sheep	:	Dr. D.E.S. Stewart-Tull, Department of Microbiology, University of Glasgow
dog, cat and chicken	:	Veterinary Hospital, University of Glasgow (from Mr. D.D. Whitelaw)
mouse, rat and rabbit	:	Animal House, Department of Microbiology, University of Glasgow
cod	:	Dr. T.H. Birkbeck, Department of Microbiology, University of Glasgow.

G. GENERAL METHODS

(i) Infection of cells and production of stock virus

The original stock virus was passaged in HeLa cells by infecting 3 day old confluent monolayers of 2 x 10^7 cells at an m.o.i. of one p.f.u. per cell using a suspension of virus in SFM. After an adsorption period of approximately 1 hour at 37°C, the overlay was discarded and replaced with SFM. Following incubation for 48 to 72 hours the overlay was removed and retained if necessary for separation of extracellular virus (see below). The monolayers were then scraped off using a sterile 1.5 inch silicone covered bar (Esco Rubber Co.) and magnet, and the cells suspended to a volume of 1.0 ml per 2 x 10^7 cells in SFM or Dulbecco's phosphate buffered saline "A" (PBS). The concentrated suspensions were sonicated on ice at 20 Kc/s for 5 one

minute cycles with one minute intervals for cooling using an M.S.E. 100 watt Ultrasonic Disintegrator. Gross debris was removed by brief centrifugation (1000 g) and the supernatant distributed in aliquots of 0.5 ml or 1.0 ml for storage at -70°C. This served as general virus stock and as a crude intracellular virus preparation. On thawing, all stock virus was sonicated before use.

For the preparation of crude extracellular virus, overlay medium was briefly centrifuged to remove cell debris and the supernatant used freshly.

For experimental infection of cells, an adsorption period of one hour at 37°C was generally employed and the virus was added in 1.0 ml volumes per petri dish. After this period the overlay was discarded, the monolayers washed with medium and 2.0 ml of SFM added. An m.o.i. of between 0.1 and 1.0 p.f.u. per cell was used.

(ii) Plaque assay of vaccinia virus

Vaccinia virus was assayed by a modification of the method of Appleyard and Westwood (1964b). Prewarmed 3.5 cm plastic petri dishes were seeded with 1.0×10^6 HeLa cells suspended in MEM containing 10 per cent calf serum and supplemented with 0.75 per cent carboxymethyl cellulose to obtain a uniform monolayer (Ross, Cameron and Wildy, 1972). The plates were incubated stacked in sealed sandwich boxes under an atmosphere of 5 per cent CO_2/air at 37°C for a period of 1-2 days or until the monolayers were completely confluent. Dilutions of test virus suspension were made in SFM, and the range of dilutions included the probable readable virus titre end point,

usually 10^{-3} to 10^{-5}. Monolayers were overlaid with 1.0 ml of each dilution (duplicate plates) and the plates reincubated at 37°C for 24 hours. On day 3, after discarding the overlay, the cell sheets were washed with a stream of SFM and overlaid with medium containing 3 per cent calf serum and 0.3 per cent rabbit anti-vaccinia hyperimmune γ-globulin concentrate to inhibit secondary spread of virus from primary foci.

The plates were reincubated at 37°C and plaques were allowed to develop over a 48 hour period. On day 5, the medium was discarded and 1.5 ml of a solution of Loeffler's methylene blue added per plate. Plates were left at room temperature for at least 30 minutes, excess stain poured off and the monolayers repeatedly washed with a stream of saline until no free dye eluted. Plaques were counted in the range 10-100. From this, the infectious virus titre of the original test virus suspension was calculated in terms of p.f.u. ml^{-1}.

(iii) Production of virus soluble antigen fractions

Soluble antigens were prepared from heavily infected cell monolayer cultures in flasks harvested 48-72 hours p.i. Cell sheets were treated by sonication as described above. However, after an initial brief centrifugation to remove gross debris, other debris and virus was pelleted by high speed centrifugation at 50,000 g for 30 minutes (MSE Superspeed 65) using 10 ml polypropylene tubes, and a 10 x 10 ml titanium angle rotor. The supernatant was retained as the soluble antigen fraction and occasionally was concentrated by dialysis (Visking tubing) against polyethylene glycol 4000 for 4 hours at 4°C.

Corresponding soluble antigen fractions were prepared from uninfected cell cultures as controls. Fractions were stored at $-20^{o}C$.

(iv) Production of antisera and preparation of γ-globulin concentrates

(a) Rabbit anti-vaccinia hyperimmune serum. A crude extract of rabbit dermal pulp was mixed 1:1 with complete Freund's adjuvant (C F A). One ml of this emulsion was injected intramuscularly (I M) per rabbit weighing approximately 2 kg and a test bleed taken 21 days later.

10 weeks after the primary injection, 0.5 ml of virus suspension, without adjuvant, was administered intravenously (I V) to each rabbit, followed by two similar injections at 2 day intervals. A test bleed was taken 6 days after the final injection.

After a total of 5 months, the rabbits were given an additional three "booster" injections as above, and bled out 5 days after the last injection.

(b) Rabbit antiserum to whole HeLa cells. HeLa cells (4×10^{7}) grown in monolayer culture were disrupted by ultrasonication and emulsified in C F A (1:1). Two New Zealand White rabbits, approximately 2 kg in weight, were injected I M with 0.5 ml emulsion. This procedure was repeated 14 days later. Booster I V injections were given at 8 and 16 weeks (0.25 ml per rabbit, without adjuvant) and the animals bled out 5 days after the final injection.

(c) Rabbit antiserum to plasma membranes from uninfected HeLa cells. Two 2 kg Lac Dutch rabbits were used; 200 μg of membrane protein from a purified HeLa cell plasma membrane preparation in 1.25 ml was

emulsified with an equal volume of C F A. This mixture (0.5 ml containing 40 μg protein) was injected I M into each rabbit. Eight weeks later, the rabbits were again injected I M with 40 μg protein from the above preparation. Five days later, 50 μg of a purified plasma membrane preparation in 10 mM Tris-HCl buffer pH 7.0 was injected I V without adjuvant. Test bleeds were made seven days later. Booster I V injections of 50 μg aliquots of plasma membrane protein were administered 6 and 8 weeks later. Rabbits were bled out by cardiac puncture five days after the final injection.

(d) Rabbit antiserum to plasma membranes from vaccinia-infected HeLa cells. Two 2 kg New Zealand White rabbits were used; 0.6 ml of a preparation of purified plasma membranes from vaccinia-infected HeLa cells was emulsified in an equal volume of C F A and 0.5 ml aliquots of the emulsion, containing approximately 40 μg of protein, were injected I M. Four weeks later the rabbits were given a second similar injection. After a further 12 days an I V booster injection of 50 μg of "infected" plasma membrane protein was given and a further injection four weeks later. Test bleeds were taken seven days after booster injections had been administered and after further "boosters". The animals were bled out by cardiac puncture five days after the last injection.

(e) Sheep antiserum to rabbit-γ-globulin. This was obtained as a γ-globulin concentrate from Dr. Stewart-Tull, Department of Microbiology, University of Glasgow (Stewart-Tull and Rowe, 1975).

All sera and concentrates were stored at $-20^{\circ}C$ until required.

(f) Preparation of γ-globulin concentrates. These were prepared by the method of Smith *et al* (1962).

(v) Immunodiffusion

This was carried out on microscope slides according to the method of Crowle (1958).

The slides were incubated at room temperature for 36 to 60 hours to allow precipitin lines to develop, after which the template was removed.

The slides were soaked overnight in PBS, stained for ten minutes in 2 per cent acetic acid/saturated nigrosin solution and destained with 2 per cent acetic acid/1 per cent glycerol and dried at 37°C.

The diffusion patterns were photographed using the slide as a negative, by projection onto photographic paper (Kodak Ltd.).

(vi) Complement-fixing titres of antisera

This was performed according to the micro-method described by Grist, Ross and Bell (1974). The antigen source was the supernatant from an extract of rabbit dermal pulp infected with vaccinia virus.

The complement-fixing titre was defined as the highest dilution of antiserum giving a reading of "4" or "3" haemolysis on a scale from 0 to 4.

(vii) Neutralising titres of antisera

Intracellular virus stock was prepared as described in "Methods" section A(i) and low titre stocks (10^4 p.f.u. ml^{-1}) were stored at $-70^{o}C$. A sample from each batch was titrated for virus and for neutralisation tests stock virus was diluted to contain approximately 200 p.f.u. ml^{-1}. 1 ml volumes of the 200 p.f.u. ml^{-1} virus stock were mixed with equal volumes of serial 10 fold dilutions of test antiserum and incubated at $37^{o}C$ for 30 minutes. For each dilution of antiserum, duplicate 0.9 ml volumes were overlaid on HeLa cell monolayers in a 25 section plastic tissue culture tray (Flow Labs Ltd.). Appropriate controls were included and plaques were counted after 3 days. The neutralising titre was defined as the highest dilution of antiserum in the initial serum-virus mixture which caused a 50 per cent reduction in plaque formation (ND_{50}).

(viii) Quantitative spectrophotometric assay of the degree of red cell binding to monolayer cell cultures

An estimation of red cell binding by quantitative measurements of haemoglobin was used in the assay for haemadsorption (HAD) and adapted for the con-A-coated red cell binding assay.

Monolayers with bound red cells were washed three times with PBS to remove non-specifically adherent erythrocytes. After drying, 2.0 ml of 0.005 per cent saponin solution in PBS was added to each plate to lyse the red cells. The entire cell sheet was scraped off into the saponin solution and transferred to a 4" x $\frac{1}{2}$" test tube. The optical density was measured against distilled water at wavelengths

of 410 and 280 nm using silica (1 cm) semi-micro cells and a Pye Unicam SP500 spectrophotometer. The $E_{410\ nm}$ gave an assessment of the haemoglobin content of the suspension but because of the gross cytopathic effects induced by vaccinia late in infection, parts of the cell sheet became detached resulting in less overall red cell binding; thus the simple measurement of $E_{410\ nm}$ was not adequate and $E_{280\ nm}$ was measured to allow corrections to be made for the detached cell sheet.

It may be assumed that the measured E_{410} (E_{410R}) is the sum of the $E_{410\ nm}$ of lysed turkey erythrocytes and HeLa cells (E_{410T} and E_{410H} respectively). Similarly, the measured E_{280} (E_{280R}) is the sum of the $E_{280\ nm}$ of lysed turkey erythrocytes (E_{280T}) and HeLa cells (E_{280H}). For the quantitative haemadsorption test the degree of binding of turkey erythrocytes to HeLa cells (E_{410T}/E_{280H}) is required and it can be shown that

$$\frac{E_{410T}}{E_{280H}} = \frac{E_{410R} - xE_{280R}}{E_{280R} - \frac{1}{y} E_{410R}}$$

$$\text{where } x = \frac{E_{410H}}{E_{280H}}, \quad y = \frac{E_{410T}}{E_{280T}}$$

Lysed HeLa cells and lysed erythrocytes consistently gave E_{410}/E_{280} ratios of 0.250 and 1.92 respectively. The derivation of the above equation is shown in Appendix 2.

(ix) Preservation of turkey red blood cells by freezing in liquid nitrogen

The citrated suspension of red cells was made 15 per cent (v/v) with respect to glycerol by addition of an equal volume of 30 per cent glycerol in PBS. The suspension was then transferred to a glass separating funnel and blood was dropped ($\sim$ 50 drops min^{-1}) from a height of 20 cm into liquid nitrogen contained in a polystyrene ice bucket. Frozen globules of blood were transferred to metal pipette canisters with drilled outlet holes and these were stored under liquid nitrogen. Cells remained usable for at least four months under these conditions. The frozen globules were found to reconstitute optimally when thawed quickly with stirring, in an equal volume of 15 per cent glycerol in warm PBS. After centrifugation, the pelleted red cells were washed once with 5 per cent glycerol in PBS and at least twice in PBS before resuspending to the desired concentration. Cells reconstituted from the frozen state could be stored for up to 4 days at $4^{o}C$.

(x) Sodium dodecyl sulphate polyacrylamide gel electrophoresis

The method of Davis (1964), as modified by McNiven, Owen and Arbuthnott (1972) was used for disc gel electrophoresis, while slab-gel electrophoresis was conducted by the method of Wardlaw, Parton and Hooker (1976).

(xi) Protein estimation

This was carried out as described by Lowry *et al* (1951).

(xii) Estimation of sucrose content of density gradient fractions

The sucrose content, in terms of percentage weight by volume was measured using an Abbé refractometer.

H. DETECTION OF VIRUS-INDUCED CELL SURFACE ANTIGENS

(i) Immunofluorescence

The appearance of VICSA was monitored by a double layer fluorescent antibody technique using unfixed cells. Test monolayers in 3.5 cm plastic petri dishes were washed three times with PBS and 0.5 ml of test antiserum added and plates were incubated for 30 minutes at 4°C. After washing three times with PBS, 0.5 ml volumes of a 1/5 dilution of fluorescein isothiocyanate (FITC)-conjugated sheep anti-rabbit immunoglobulin were added to each plate and allowed to react for 30 minutes at 4°C. Monolayers were then washed five times with PBS to remove excess conjugate and examined by phase contrast and by U V light for specific fluorescence (Leitz Orthoplan; E. Leitz, London) using fluorescein filters (BG 12, 2 x FITC Filters) and a Fluorescence Vertical Illuminator (Ploem, 1967). Sample fields (phase contrast and ultraviolet) were photographed using an automatic exposure device and black and white Kodak film (FP4, ASA125, DIN22) developed in Kodak Microdol-X and fixed in Ilford Hypam.

(ii) Immune haemadsorption

This was based on the method described by Miyamoto and Kato (1968).

(a) Preparation of complete indicator cells. Doubling dilutions (0.1 ml) of rabbit anti-sheep red cell haemolysin, inactivated at 56°C for 30 minutes, were made in PBS in a Cooke Microtiter plastic disposable tray and 0.1 ml volumes of a 4 per cent washed sheep red cell

suspension were added per well. The tray was shaken, incubated at 37°C for 60 minutes and read after 2 hours further incubation at room temperature. The dilution of antiserum which just failed to agglutinate was noted. The antiserum was diluted accordingly and equal volumes of dilute antiserum and 4 per cent red cell suspension were mixed and incubated at 37°C for 30 minutes with occasional agitation. The sensitised red cells were centrifuged and washed twice in PBS before resuspending to 2 per cent.

Goat anti-rabbit precipitating serum (inactivated and absorbed 1:1 with packed sheep red cells) was titrated in doubling dilutions as described above, against the 2 per cent sensitised sheep erythrocytes. Control dilutions were also titrated against unsensitised red cells. The highest dilution just failing to give maximum agglutination was recorded. The goat anti-rabbit serum was diluted appropriately and mixed with 2 per cent sensitised sheep cells in equal volumes, and the suspension incubated at 37°C for 30 minutes, after which the cells - "complete indicator cells" - were pelleted and washed twice in PBS before suspension to 0.5 per cent in PBS for storage at 4°C.

(b) Assay of complete indicator cells. Rabbit anti-HeLa cell serum (inactivated) was diluted 1/20 in PBS and absorbed with packed sheep red cells (1:1) for 30 minutes at 37°C. Monolayer cultures of uninfected HeLa cells in 3.5 cm plastic petri dishes were washed with PBS, overlaid with 0.5 ml of the absorbed antiserum and incubated at 37°C for 30 minutes. Monolayers were washed three times with PBS, treated with 0.5 ml of complete indicator cells (CIC) or a 0.5 per cent suspension of sensitised sheep red cells for 30 minutes at 37°C.

Control plates were also prepared using inactivated absorbed normal rabbit serum. After washing off excess red cells, monolayers were examined for specific binding of CIC or sensitised red cells, either by microscopy or by the quantitative haemadsorption method.

Batches of CIC showing positive adsorption, in the absence of absorption of sensitised red cells, were used in testing for immune haemadsorption.

(c) Assay for virus-induced cell surface antigens by immune haemadsorption. The assay was conducted as above except that rabbit anti-HeLa cell serum was replaced by rabbit anti-vaccinia γ-globulin concentrate (antiVACγG) diluted 1/10 to 1/20, and absorbed with sheep red cells. Other inactivated antisera were also tested. The bound red cells were lysed and the degree of binding assessed spectrophotometrically. However, since, in these experiments, minimal loss of cells from test monolayers occurred, only the $E_{410\ nm}$ of the lysate was recorded.

(iii) Complement fixation

The assay was designed to test for the presence of VICSA by measuring any depletion in the amount of added complement when antiVACγG was incubated with virus-infected cell monolayers. Fixation of complement would thus indicate the presence of cell surface viral antigens.

By carrying out a "chessboard" titration of complement and rabbit anti-sheep red cell haemolytic serum against 4 per cent washed

sheep red cells in veronal-buffered saline (VBS) (0.25 ml volumes), the optimal sensitising dose of haemolytic serum, i.e. that giving complete lysis with the highest dilution of complement, and the highest dilution of complement giving 50 per cent lysis (HD_{50}) were determined. Appropriate controls were included. In the subsequent test 4 HD_{50} units of complement were used per test plate.

Monolayers in 3.5 cm plastic petri dishes were tested at various times after infection. The cell sheet was washed with VBS and treated for 20 minutes at $37^{o}C$ with 0.5 ml per plate of a 1/10 dilution in VBS of either normal rabbit γ-globulin (NγG) or rabbit antiVACγG concentrates. The monolayers were then washed three times with VBS and 4 HD_{50} units of complement, in a volume of 0.5 ml, added. Incubation was at $37^{o}C$ for 20 minutes, after which all the complement overlay was carefully removed and the plates discarded. The complement fractions were retitrated (using VBS as diluent and perspex WHO trays), against washed 4 per cent sheep red cells which had been treated with the optimal sensitizing dose of haemolytic serum. The results were read after 40 minutes at $37^{o}C$.

(iv) Complement-mediated cytolysis

The method employed was based on the "dye exclusion" assay of Saijo (1973) and was carried out using two approaches.

Test monolayers in 3.5 cm plastic petri dishes were washed with the buffer described by Saijo - termed here "Saijo" buffer. Gelatin was omitted from the buffer. Concentrates of rabbit NγG and

antiVACγG were diluted in "Saijo" buffer to contain 1-2 mg protein ml^{-1} and 0.75 ml volumes were overlaid on test plates which were incubated for 30 minutes at 37°C before washing three times with buffer.

Reconstituted freeze-dried guinea-pig complement was diluted 1/20 in "Saijo" buffer and 0.75 ml per plate was added. The plates were incubated at 37°C for 30 minutes and transferred to a -20°C freezer to bring the temperature quickly to $\sim$ 0°C to inhibit further complement activity. Ice-cold 1 per cent trypan blue in buffer (0.2 ml per plate) was added to each plate and mixed with the complement overlay. The plates were incubated for a further 7 minutes, washed four times with "Saijo" buffer, excess moisture dried off and the cell layer solubilised by the addition of 2.0 ml of 1M NaOH. After vortexing, the $E_{580}^{1\ cm}$ of the solubilised cells was determined (Pye Unicam SP500 Spectrophotometer, silica semi-micro cells).

An alternative method was devised which proved more sensitive. Instead of pretreatment of test monolayers with normal or antiviral γG, this was added simultaneously with complement. Otherwise the assay was as described above.

(v) ^{125}Iodine-labelled antibody binding

Two volumes of hyperimmune sheep anti-rabbit serum were mixed with one volume of saturated ammonium sulphate solution and the resulting precipitate dissolved in saline and dialysed against PBS. The final protein concentration was 15 mg ml^{-1}. Iodination of the γ-globulin concentrate was carried out by a modification of the method of McConahey and Dixon (1966). One mCi (10 μl) of carrier-free Na ^{125}I

and 200 μl of chloramine-T solution (1 mg ml^{-1}) were added dropwise to 100 mg of γ-globulin concentrate in a volume of <2.0 ml. After 5 minutes the reaction was terminated by addition of 200 μl of sodium metabisulphite (1 mg ml^{-1}) and 200 μl of potassium iodide (5 mg ml^{-1}). The reaction mixture was applied to a column of Sephadex G-25 to separate unreacted Na ^{125}I and iodinated γ-globulin. Fractions containing coincident peaks of ^{125}I and protein were pooled and stored at $4^{o}C$.

Binding assay. Monolayers of HeLa cells on 3.5 cm plates were washed with PBS before treatment with dilutions of rabbit NγG or antiVACγG (0.75 ml) for 30 minutes at $37^{o}C$. The plates were washed three times with PBS and incubated with 0.5 ml volumes of ^{125}I-labelled sheep anti-rabbit globulin (^{125}I-SARγG) at varying concentrations. After 30 minutes at $37^{o}C$, the monolayers were rinsed six times with PBS and cells brought into suspension by treatment with 0.25 per cent trypsin in PBS. The trypsinates were transferred to glass scintillation vials and ^{125}I was determined using a Nuclear Enterprises Liquid Scintillation Counter (model 8312).

I. ASSAYS OF VACCINIA HAEMAGGLUTININ

(i) Assay for haemadsorption of susceptible red blood cells to virus-infected cells

This assay measured the binding of turkey red cells to the infected cell surface by VHA. Infected cell monolayers in 3.5 cm plastic petri dishes were washed with SFM and a 5 per cent suspension of turkey red cells in PBS added (0.75 ml per plate). The plates

were incubated stationary at 37°C for 30 minutes to allow settling and binding, washed with PBS and the bound red cells assayed as described previously.

(ii) Assays for free haemagglutinin and haemagglutination inhibition

"Soluble" haemagglutinin in infected cell extracts was assayed in plastic disposable Cooke Microtiter trays. Serial 0.1 ml doubling dilutions of test suspension were made in PBS containing 1 per cent normal rabbit serum as an inhibitor of non-specific agglutination (Burnet and Stone, 1946); 0.1 ml per well of 1 per cent turkey red cells in 1 per cent normal rabbit serum/PBS was added. The plates were agitated briefly and incubated at 37°C for 60 minutes with occasional agitation. After further incubation for 60 minutes at room temperature the plates were read. Complete red cell shielding was scored as 5+, partial as 4+, granular agglutination as 3+, moderate granular agglutination as 2+, slight agglutination as 1+ and no agglutination as 0. The 50 per cent end point was taken as the last well giving 3+ haemagglutination and the inverse of this dilution was designated the HA_{50} titre.

In the HAI assay, 0.1 ml doubling dilutions of test serum were made in 1 per cent normal rabbit serum/PBS in a Microtiter plate. A standard suspension of VHA (an extract of virus-infected cells) was diluted to contain 4 HA_{50} units per 0.1 ml and 0.1 ml added per well. Plates were incubated at 37°C for 30 minutes, 0.1 ml of 1 per cent turkey red cells in 1 per cent normal rabbit serum/PBS was added per well and the plates incubated and read as in the HA test. The

reciprocal of the highest dilution of antiserum reducing haemagglutination to 3+ was defined as the HAI_{50} titre.

(iii) Purification of vaccinia haemagglutinin

This was carried out by the method of Gurvin and Haukenes (1973); 0.1M glycine·NaOH buffer pH 8.6 was used for all solutions. From 48 h-infected cells the overlay was discarded and the cell sheet scraped into glycine-NaOH buffer using a silicone covered bar and magnet to a final volume of 2.5 ml per 2×10^7 cells. The infected cell suspension was frozen and thawed twice and sonicated at 20 Kc/s in an MSE 100 watt Ultrasonic Disintegrator at $0^{\circ}C$ for 120 s. After centrifugation (1000 *g* for 10 min) the supernatant was made up to 10 per cent w/w with sucrose. The 10 per cent sucrose supernatants were overlaid on to 40 per cent w/w sucrose in a 25 ml polycarbonate centrifuge tube using a volume-volume ratio of 7:3. Gradients were centrifuged at 90,000 *g* for 12 h, using a 3 x 25 ml aluminium swing-out rotor in the MSE Superspeed 65. Gradient tubes were pierced from the bottom (MSE Tube Piercer) and fractions of 50 drops (∿ 1.0 ml) collected and tested for sucrose concentration, HA activity and protein content. Fractions containing the bulk of HA activity were pooled, adjusted to 50 per cent w/w with respect to sucrose, transferred to a 12 ml polycarbonate centrifuge tube and overlaid with 40 per cent w/w sucrose (volume ratio 3:4). This gradient was centrifuged at 100,000 *g* for 18 h as described above and fractions of 10-20 drops were collected and assayed as before.

J. DETECTION OF VIRUS INDUCED CELL SURFACE CHANGES

(i) Concanavalin A agglutination of vaccinia-infected cells

This was based on the method of Zarling and Tevethia (1971). Human embryonic lung (HEL) cells were infected with vaccinia and tested at predetermined periods after a one hour adsorption period. Cells were detached from duplicate petri dish cultures by treatment with 0.02 per cent ethylenediaminetetra-acetic acid (EDTA) in PBS, washed twice in PBS and resuspended in normal saline (1.0 ml per plate culture). From this suspension, duplicate 0.1 ml volumes were mixed with 0.1 ml saline, while a further two 0.1 ml volumes of suspension were mixed with 0.1 ml volumes of con A (1000 $\mu g\ ml^{-1}$ in saline). After 15 minutes incubation at room temperature, the droplets were examined by inverted microscope for agglutination.

(ii) Binding of concanavalin A by vaccinia-infected cells

The assay was based on a method described by Furmanski, Phillips and Lubin (1972) in which Concanavalin A binding sites on cells were measured indirectly by coating human "O" red blood cells with con A and reacting the treated cells with vaccinia-infected or uninfected HeLa cell monolayers.

Human "O" red cells were washed three times in PBS and 1.5-2.0 ml of packed erythrocytes were resuspended in 10 ml of a 200 $\mu g\ ml^{-1}$ solution of con A in PBS. The optimum concentration of con A for coating was determined by assay (see "Results" p 112). The suspension was incubated with occasional agitation at 37°C for 60 minutes

and the coated cells washed thrice in PBS before resuspension to 2 per cent in PBS. Test HeLa cell monolayers in 3.5 cm plastic petri dishes were washed with PBS and overlaid with 1.0 ml of the conA/red cell suspension. Plates were incubated at room temperature for 20 minutes, excess unbound red cells washed off with PBS and the amount of bound red cells estimated by the quantitative haemadsorption test.

(iii) Assay of total sialic acid in vaccinia infected cell extracts

Sialic acid was estimated using the method of Warren (1959) (see Appendix 5).

K. HELA CELL PLASMA MEMBRANES : PREPARATION, PURIFICATION AND CHARACTERISATION

(i) Method of preparation and purification

This was based principally on the methods described by Atkinson (1973) and Johnsen, Stokke and Prydz (1974). Normally 2 to 8 x 10^7 cells were processed in one batch from monolayer cultures grown in 120 cm^2 glass flasks. Uninfected cells were harvested at 3 to 6 days after seeding or after reaching confluence. Virus-infected cells were harvested at 24 hours p.i.

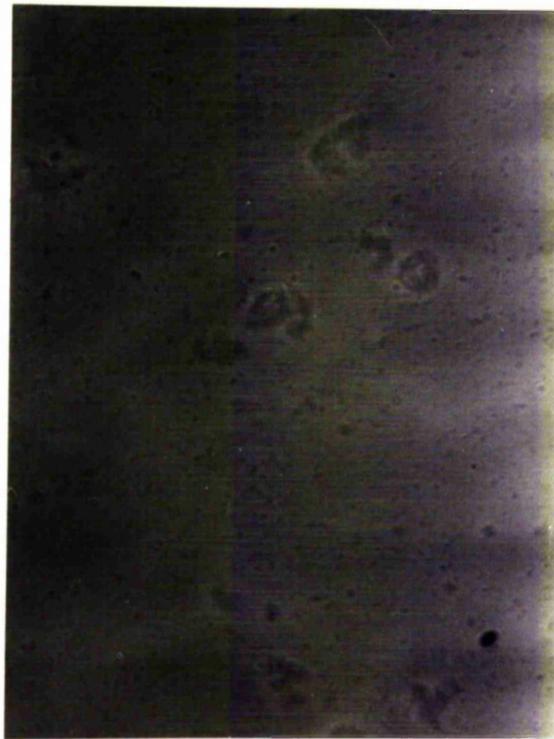
After decanting the overlay medium, the monolayers were washed with PBS; 10 ml of 0.02 per cent EDTA in PBS was added, and cells incubated at 37°C for 20 minutes. The detached cells were collected by centrifugation and washed once in PBS, twice in cold 10 mM Tris-HCl buffer (pH 7.0) and once in cold 10 mM Tris-HCl pH 8.0. The cells were finally allowed to swell in 5 ml of 10 mM Tris-HCl pH 8.0 for 10 min at 0°C.

The swollen cell suspension was transferred to a pre-cooled 5 ml glass homogenisation vessel (Jencons Uniform, body type PTG 2). Efficient breakage was achieved with a plunger giving a clearance of 0.05 mm. A series of Teflon "bell-shaped" plunger heads were prepared by the Mechanical Engineering Department, Research Annexe, University of Glasgow. Two full strokes were found to be sufficient for breakage of 95 per cent of cells as monitored by phase contrast microscopy. Cold 2 mM calcium acetate in 10 mM Tris-HCl pH 7.0 (5 ml) was slowly added to stabilise the free nuclei which became refractile with the nucleoli visible. The ghosts appeared at this stage as limp, transparent "leaves" (Plate 1).

The suspension was transferred to a conical centrifuge tube and centrifuged at 680 g for 90 s to deposit nuclei and unbroken cells. If required for enzyme assay, the pelleted nuclei were resuspended in 2.0 ml 2 mM calcium acetate/10 mM Tris-HCl pH 7.0. The 680 g supernatant, containing substantial numbers of ghosts in diluted cytoplasm, was subjected to sucrose density gradient centrifugation. Supernatant (3.5 ml) was layered on top of a discontinuous sucrose gradient comprised of 1.5 ml 45 per cent w/v sucrose/10 mM Tris-HCl pH 7.0 in the bottom layer, and 4.0 ml of 30 per cent w/v sucrose in the same buffer, in the top layer. Gradients were formed in 12 ml polycarbonate tubes (MSE) and were centrifuged at 10,000 g for 20 min at 4°C, in a 3 x 25 ml aluminium swing out rotor with adaptors and an MSE Superspeed 65 centrifuge. Two distinct bands formed; one, at the 30 per cent w/v sucrose/sample interface, was composed, as judged by microscopy, of cytoplasmic organelles and very small membraneous

<u>Plate 1</u> <u>HeLa cell plasma membrane "ghosts"</u>

Plasma membranes, prepared as shown in Figure 11 , were photographed under phase contrast at a magnification of X400. Plate magnification 2.8X.



structures; the second, at the 30 per cent/45 per cent w/v sucrose interface, was composed of large, intact ghosts. An ill-defined zone between the interfaces may have represented fragmented plasma membrane material.

The two bands were extracted from the top of the gradient with a syringe and 2" needle. The first, designated "top" band, was retained for enzyme assay. The second band was diluted 1:3 in 10 mM Tris-HCl pH 7.0, applied to another 30 per cent/45 per cent sucrose gradient and recentrifuged as described. The band formed from this centrifugation, at the 30 per cent/45 per cent interface, was extracted, diluted 1:8 in buffer and centrifuged again at 10,000 g for 5 min. The membranes were recovered in the pellet and resuspended in buffer (1.0 to 2.0 ml) for assay and examination by microscopy.

(ii) Enzyme assays

(a) Assay of potassium-dependent phosphatase activity of cell fractions. The enzyme activity was measured by the method of Robinson (1975) and monitored the release of nitrophenol from p-nitrophenyl phosphate (Tris salt).

The reaction mixture contained:-

	Final concentration
1.0 ml 60 mM Histidine monohydrochloride-Tris buffer, pH 7.8	30 mM
0.5 ml 12 mM $MgCl_2$	3 mM
0.3 ml 20 mM p-nitrophenyl phosphate (Tris salt)	3 mM
0.2 ml test suspension.	

A parallel reaction mixture was also prepared with a solution of 12 mM $MgCl_2$ 40 mM KCl replacing 12 mM $MgCl_2$. The p-nitrophenyl phosphate was added last to initiate the reaction, and the assay tubes incubated in duplicate for 30 min in a 37°C water bath. The reaction was stopped by the addition of 2.0 ml of 2M NaOH and the extinctions read at 410 nm against distilled water in a Pye Unicam SP500 spectrophotometer. Readings were compared in the presence or absence of K^+ ions.

(b) Assay of phosphodiesterase activity in cell fractions. Phosphodiesterase was measured by the method of Ostrowski and Tsugita (1961). The reaction mixture contained:-

0.5 ml 0.1M Tris-HCl buffer pH 8.9 containing

~~0.4 ml~~ 0.02M $MgSO_4$

0.4 ml 0.025M Calcium bis p-nitrophenyl phosphate

0.1 ml of test suspension.

Duplicate assay tubes were incubated at 37°C for 30 minutes and the reaction terminated by addition of 1.0 ml 0.1M NaOH. Tubes were centrifuged at 1000 g for 15 minutes and the $E_{410\ nm}$ read against distilled water as blank reference (Pye Unicam SP500 Spectrophotometer, semi-micro silica cuvettes, 1 cm path length).

(c) Assay of NADH diaphorase activity in cell fractions. The method of Wallach and Kamach (1966) was employed.

The assay mixture consisted of:-

0.4 ml of 0.02M Tris-HCl buffer pH 7.4

0.2 ml of 0.0033M potassium ferricyanide

0.4 ml of a freshly prepared solution of NADH, 100 µg ml^{-1} in 0.02M Tris-HCl buffer pH 7.4.

This was transferred to a 1 cm silica semi-microcuvette and the reaction started by addition of 0.1 ml of test suspension activated at 37°C for 2 minutes. The oxidation of NADH was followed using a Pye Unicam SP500 spectrophotometer by measuring the decrease in $E_{340\ nm}$ when read against distilled water in the reference cell. The reaction rate, linear over 2 to 3 minutes, was measured at this stage in all fractions and average rates compared graphically.

(d) Assay of β-glucuronidase activity in cell fractions. This was performed by the method of Allison and Sandelin (1963).
The assay mixture consisted of:-

0.5 ml of 0.1M sodium acetate buffer

0.1 ml of 0.01M phenolphthalein β-glucuronide

0.1 ml of test suspension.

The mixtures were incubated in duplicate in a 37°C water bath for 30 min and the reaction stopped by addition of 2.0 ml 0.4M glycine-NaOH buffer pH 10.7 which provided the alkaline conditions for development of the pink colour of free phenolphthalein. The suspensions were centrifuged at 1200 g for 15 min in a bench centrifuge and the $E_{545\ nm}$ of the supernatants read against distilled water.

(iii) Measurement of specific uptake of ^{3}H-L-fucose by plasma membranes

The method described by Atkinson (1973) was followed.

1.0×10^{7} exponentially growing cells were overlaid with SFM containing 80 μCi ^{3}H-L-fucose and incubated for 24 hours at 37°C.

The overlay was removed and stored, and the monolayer washed thoroughly with PBS. The total "wash" volume was also stored. Cells were then processed as described above to produce a purified plasma membrane fraction. Fractions were assayed for the uptake of ^{3}H-L-fucose by two methods:

(a) Direct assay. 200 μl volumes were pipetted into glass scintillation vials and 4.0 ml of scintillant fluid (NE260 Micellar Scintillator, Nuclear Enterprises Ltd.) added. Protein estimations were carried out on these fractions.

(b) Assay of acid precipitable radioactive material. 100 μl volumes were pipetted onto 2.0 cm^2 pieces of filter paper (Whatman No. 1). These were air dried and immersed for 60 minutes in 10 per cent ice-cold trichloroacetic acid (TCA), 20 minutes in 5 per cent cold TCA and finally in cold 95 per cent ethanol for 20 minutes. The filter papers were placed in glass scintillation vials and 8.0 ml of scintillant fluid added. The radioactivity was counted using a Nuclear Enterprises Liquid Scintillation Counter (model 8312).

RESULTS

A. DETECTION OF VIRUS-INDUCED CELL SURFACE CHANGES : ANTIGENIC CHANGES

Previous work employing immunofluorescence and immune haemadsorption had indicated the presence of specific "early" VICSA on the poxvirus-infected cell surface (Miyamoto and Kato, 1968, 1971; Ueda et al, 1969) and other VICSA which increased in quantity during infection (Brier et al, 1971; Hayashi et al, 1972). In the present work, attempts were made to demonstrate VICSA using a range of techniques.

(i) Immunofluorescence

Vaccinia-infected HeLa cell monolayers were tested at 2 and 24 h p.i. for the presence of VICSA using specific rabbit antiVACγG followed by FITC-labelled sheep anti-rabbit γG. Cells infected in the presence of ara-C (25 μg ml^{-1}) were similarly tested for VICSA.

In a preliminary experiment to test the specificity of staining, monolayers of uninfected HeLa cells treated with anti-HeLa cell serum followed by FITC-labelled conjugate showed strong surface fluorescence (Plate 2). Control plates treated with PBS or normal serum prior to addition of conjugate showed no fluorescence indicating the specificity of the staining. Virus-infected cells tested with anti-VACγG at 2 h p.i. displayed whole cell fluorescence (Plate 3b) which by 24 h p.i. had only moderately increased (Plate 3d). Cells infected in the presence of ara-C showed similar fluorescence by 24 h p.i. when tested with anti-VACγG (Plate 3f). Virus-infected cells treated with either PBS or normal serum followed by FITC-sheep anti-rabbit γG showed no fluorescence.

Plate 2 Immunofluorescence of uninfected HeLa cells

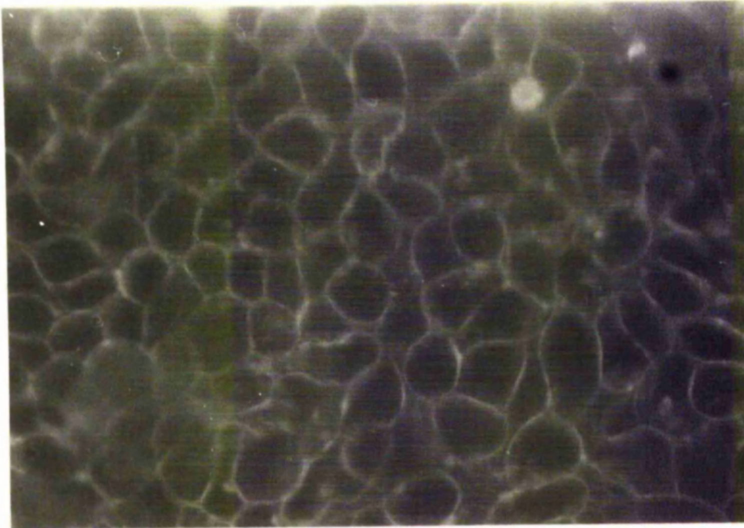
Confluent monolayers of unfixed HeLa cells were incubated with rabbit anti-HeLa antiserum, washed and further incubated with FITC-labelled sheep anti-rabbit globulin. After washing in PBS, the cells were examined for fluorescent staining and the same field photographed under UV light and phase contrast.

Magnification: 400X, Plate magnification 2.8X.

Plate 2a:- phase contrast

Plate 2b:- UV

a



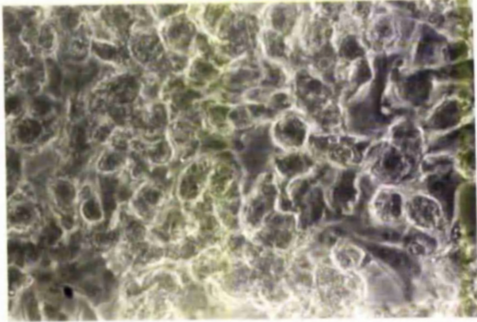
b

Plate 3   Immunofluorescence of vaccinia-infected HeLa cells

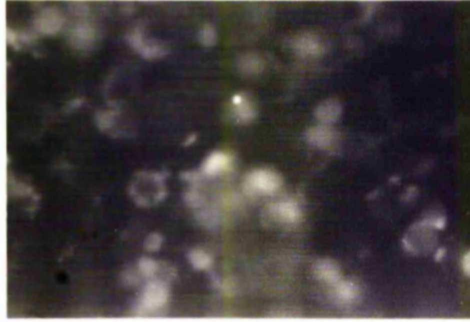
Confluent monolayers of infected HeLa cells were incubated with rabbit anti-vaccinia antiserum. After washing in PBS, the monolayers were treated with FITC-labelled sheep anti-rabbit globulin and again washed in PBS. The cells were examined for fluorescent staining under UV light and by phase contrast.

Magnification: 400X, Plate magnification 1.8X.

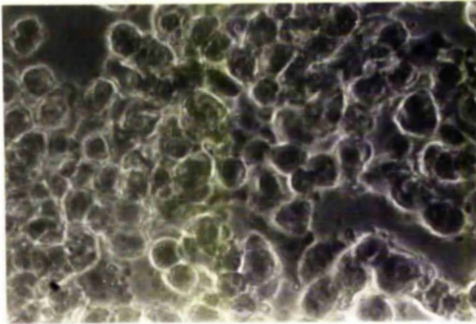
Plate 3a:-	2 h	p.i.;	no ara-C;	phase contrast
" 3b:-	"	"	" "	UV
" 3c:-	24 h	"	" "	phase contrast
" 3d:-	"	"	" "	UV
" 3e:-	"	"	+ ara-C;	phase contrast
" 3f:-	"	"	"	UV



a

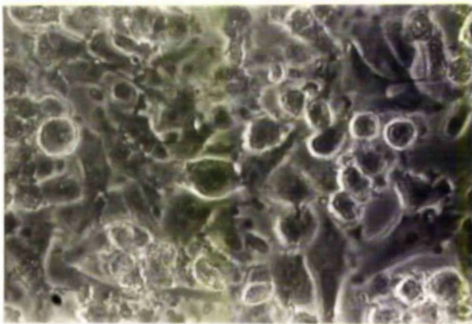


b

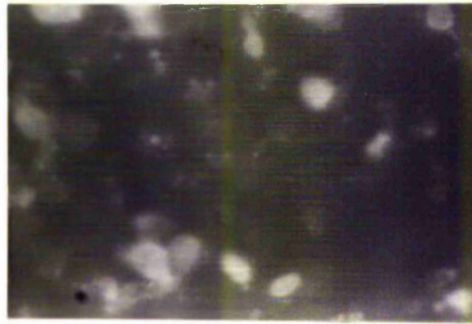


c

d



e



f

(ii) Immune haemadsorption

In the present work VICSA was readily detected by immune haemadsorption and a quantitative assay was developed based on the quantitative haemadsorption (QHAD) test ("Methods" page 54). The specificity of the reaction was tested by exposing monolayers of HeLa cells to PBS, rabbit NγG, rabbit anti-HeLa serum or rabbit anti-VACγG before addition of complete indicator cells (CIC). HeLa cells and adherent erythrocytes were lysed and the degree of red cell binding determined spectrophotometrically in terms of released haemoglobin in the lysate.

Infected monolayers were assayed at 2 h p.i. and 24 h p.i. using the same antisera. The results (Table 7) are corrected for a slight general increase in the binding of each CIC batch between the sampling times. Highly specific binding by anti-HeLa serum (inactivated) demonstrated the suitability of the test system. The test antibody, antiVACγG, was bound preferentially to virus-infected cells by 2 h p.i. and binding increased by 24 h p.i. Buffer and NγG effected no binding to uninfected or vaccinia-infected cells.

(iii) Complement fixation

This experiment was designed to establish whether the reaction between antiviral antibodies and VICSA *in situ* resulted in detectable fixation of complement.

The results (Table 8) showed a small but consistent depletion of complement at 2, 5 and 24 h p.i. when antiVACγG but not

Table 7 Detection of vaccinia-specific antigens on the surface of infected HeLa cells assayed by immune haemadsorption

Monolayers were tested at 2 and 24 h p.i. by the addition of PBS or one of the listed sera followed by CIC and the degree of red cell binding estimated quantitatively, by measuring haemoglobin in the cell lysate ($E_{410\ nm}$).

Serum treatment	Average $E^{1\ cm}_{410nm}$ of lysate (corrected)			
	Uninfected cells		Vaccinia infected cells	
	2 h	24 h	2 h	24 h
none (PBS)	0.165	0.175	0.175	0.188
NγG	0.172	0.184	0.161	0.182
anti-HeLa serum	1.050	1.070	n.t.	n.t.
anti-VAC γG	0.165	0.184	0.285	0.600

n.t. = not tested

Table 8 Fixation of complement by vaccinia-induced cell surface antigens in the presence of antiviral γ-globulin: titration of residual complement

Following incubation with antibody, test monolayers were washed and allowed to react with guinea pig complement. The overlay was later removed and residual complement determined by titration against sensitised sheep red cells. The titre was defined as the highest dilution of complement giving 50 per cent haemolysis. The working solution of complement contained 4 HD_{50} units and the results are expressed in terms of the measured number of HD_{50} units remaining in the overlay from each test plate.

		HD_{50} units per assay remaining after		
Cells	serum treatment	2 h	5 h	24 h
uninfected	normal γG	4.0	4.0	4.0
uninfected	antivaccinia γG	4.0	4.0	4.0
infected	normal γG	4.0	4.0	4.0
infected	antivaccinia γG	2.7	2.7	1.8-2.7

NγG reacted with vaccinia-infected monolayers. An increase in complement fixation was not detectable throughout the sampling time and the small difference observed made routine quantitative assay impracticable.

(iv) Complement mediated cytolysis

The appearance of VICSA was monitored by the complement-mediated destruction of the infected cell surface after interaction with antiviral antibody, thus allowing access of the dye, trypan blue, to the cell cytoplasm.

Initial experiments indicated that cytolysis was more pronounced when complement and antibody were added simultaneously rather than sequentially. In terms of dye uptake, cytolysis was 26 per cent greater but control monolayers and NγG treated monolayers showed no non-specific increase in uptake of dye under these conditions.

By 2 h p.i. vaccinia-infected cells took up dye in the absence of specific antibody (Fig 1). Dye uptake was slightly greater at 2 and 5 h p.i. with anti-VACγG than with NγG and by 24 h p.i. the difference in dye uptake had increased approximately fourfold from the corresponding figures at 5 h p.i., possibly reflecting an increase in the number of viral antigens expressed at the cell surface. Non-specific dye uptake also increased up to 24 h p.i. perhaps as a result of progressive virus-mediated cell death.

(v) ^{125}Iodine labelled antibody binding

Despite manipulating the conditions of preparation of

Figure 1 Detection of virus-induced cell surface antigens by complement-mediated cytolysis

Vaccinia-infected HeLa cells were examined at various times after infection, to quantitate VICSA, measured by the uptake of trypan blue after the interaction of antiviral antibody, VICSA and complement.

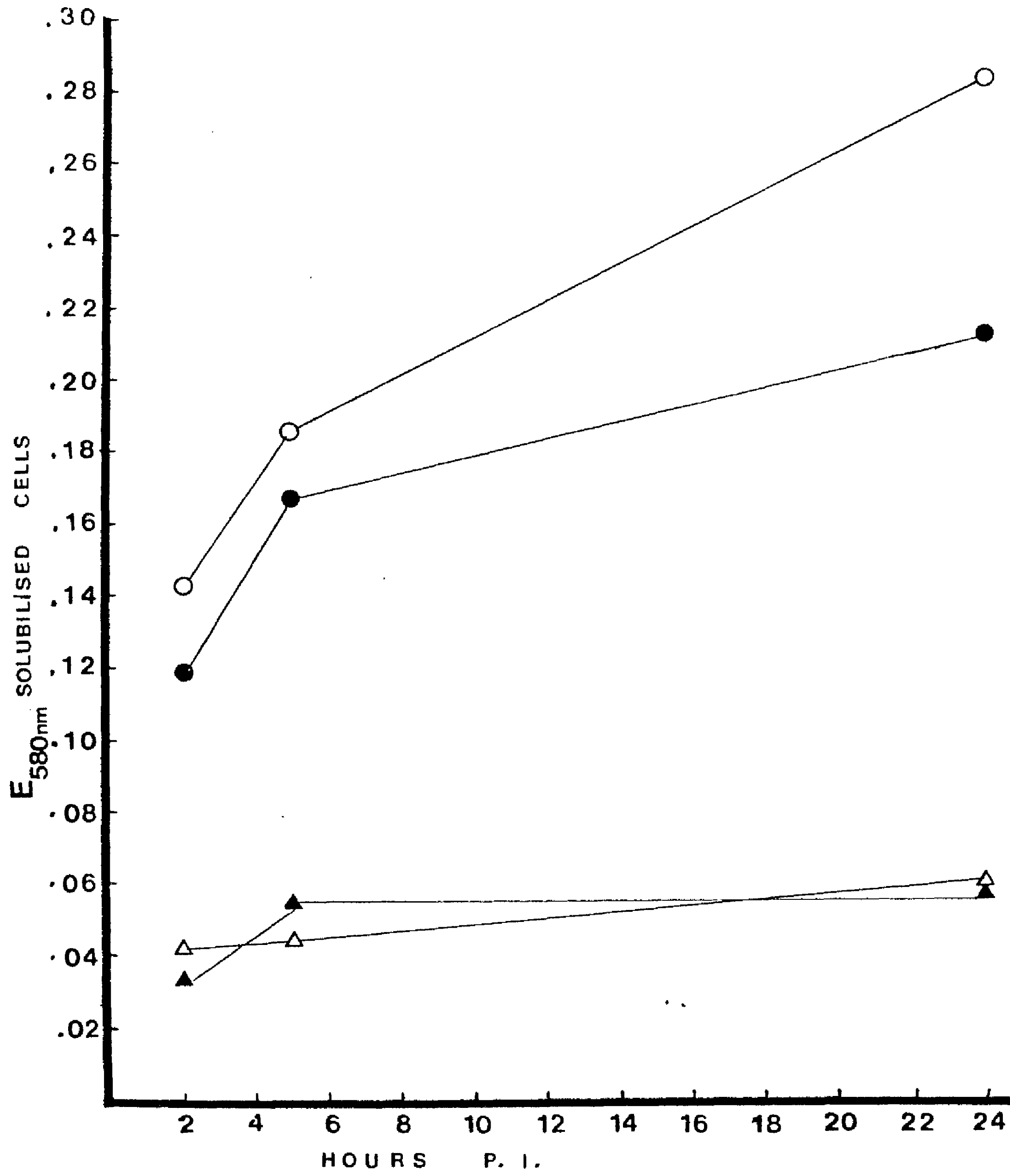
Key:-

●—● vaccinia-infected cells + NγG

○—○ vaccinia-infected cells + antiVACγG

▲—▲ uninfected cells + NγG

△—△ uninfected cells + antiVACγG


.30
.28
.26
.24
.22
.20
.18
.16
.14
.12
.10
.08
.06
.04
.02
E580nm SOLUBILISED CELLS
2
4
6
8
10
12
14
16
18
20
22
24
HOURS P. I.

^{125}I-SARγG, at no stage was preferential binding of antibody to vaccinia-infected cells detectable. In early experiments, the lactoperoxidase method of iodination was employed (Gow and Wardlaw, 1975). In common with the findings of Pagés, Louvard and Lazdunski (1975) however, a lack of ^{125}iodine incorporation into the SARγG was apparent using this method. The chloramine-T method of McConahey and Dixon (1966) yielded an improved incorporation of label. However the ^{125}I-SARγG failed to show improved or specific binding to test monolayers. To establish whether iodination had a detrimental effect on the antigen binding sites on the immunoglobulin, the specificity of ^{125}I-SARγG was tested by passive haemagglutination of tanned sheep red cells coated with normal rabbit γ-globulin. The results indicated that the iodinated antibody was still specific although the activity had been reduced by 75 per cent. This preparation incorporated 36 per cent of the $^{125}I^-$ originally added. A second preparation of ^{125}I-SARγG similarly failed to react in the antibody binding assay but was viable by the passive haemagglutination test (titre 1/1600) and had a specific activity of 1.33×10^9 counts mg^{-1} protein.

Using an antigen-antibody system consisting of human serum albumin and rabbit anti-human serum albumin in optimum proportions, ^{125}I-SARγG was found to bind to the complex. From 0.1 ml of ^{125}I-SARγG (second preparation) a maximum of 4×10^4 c.p.m. was bound to the antigen-antibody complex indicating that 2.5 per cent of the ^{125}I-SARγG protein was specific anti-rabbit γG antibody.

B. POXVIRUS HAEMAGGLUTININ

(i) Kinetics of appearance of cell surface VHA

To correlate the time of appearance of cell surface VHA with virus replication, test plates were assayed at various times after infection for a) infectious virus titre (intracellular)

b) haemadsorption

c) haemagglutinin.

The infectious virus titre rose exponentially after 10 h p.i. at the completion of one virus infection cycle (Figure 2). The HAD titre rose in parallel with the infectious virus titre but with a lag of 2 h and was still increasing by 48 h p.i. The failure of the HA assay to detect low VHA levels reflects the different sensitivity from the HAD assay. Very low levels of VHA activity ($<$ 50 per cent agglutination) were detected by 10 h p.i. but did not reach a significant level until 6 hours later.

The appearance of VHA at the cell surface was therefore characteristically that of a late viral antigen, possibly, considering the almost parallel rise, governed by the appearance of mature virus.

(ii) The effect of multiplicity of infection on cell surface VHA formation

Over the range tested (Figure 3) there was a small but measurable amount of red cell binding with m.o.i. as low as 0.01, which increased sharply as the m.o.i. was increased to 1. When an m.o.i. of 5 was employed, the consequent destruction of the cell

Figure 2 Vaccinia haemagglutinin : kinetics of appearance of cell surface VHA and intracellular virus

Vaccinia-infected HeLa cells were examined at various times after infection, for the presence of VHA by HA titration of cell extracts, for VHA at the cell surface by HAD, and for the infectious virus content by plaque assay.

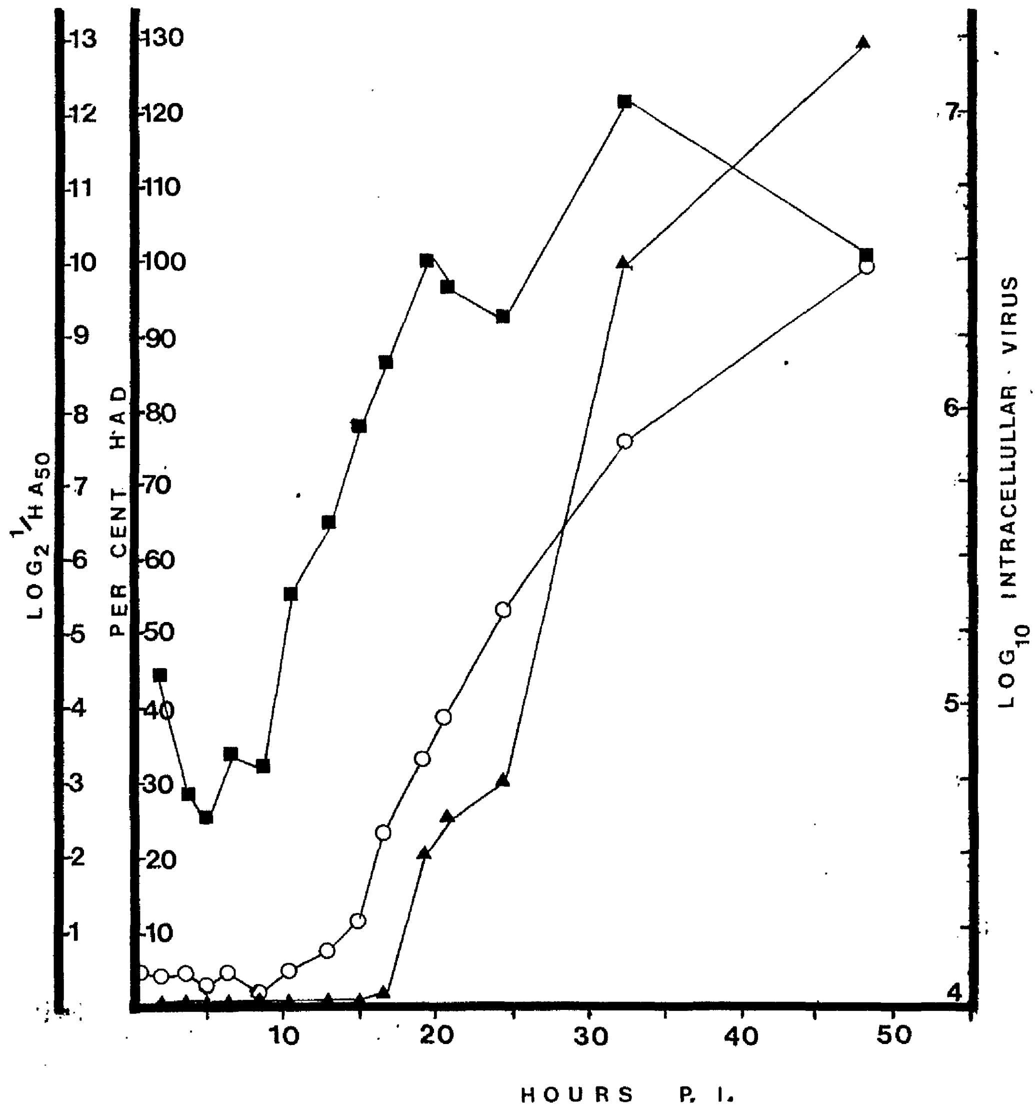
Key:-

○—○ haemadsorption

■—■ infectious virus (p.f.u.ml^{-1})

▲—▲ haemagglutinin

percentage HAD compared to 48 h-vaccinia-infected cells



LOG2 1/HA50
PER CENT HAD
LOG10 INTRACELLULAR VIRUS
HOURS P. I.

monolayer by 24 h p.i. made accurate quantitation of HAD difficult. An m.o.i. less than or equal to 1 was therefore used in subsequent experiments.

(iii) The effect of metabolic inhibitors on the expression of cell surface VHA

The effect of various metabolic inhibitors on HAD to vaccinia-infected cell monolayers was examined. After the initial virus adsorption period of 1 h, the adsorption medium was replaced with SFM containing inhibitor. At 24 h p.i. plates were assayed for HAD (Table 9) and intracellular virus titre was assessed.

All antiviral agents tested markedly inhibited infectious virus formation by at least 90 per cent and HAD was impaired by all inhibitors. Cytosine arabinoside which is an inhibitor of viral DNA synthesis, reduced HAD to background levels while hydroxyurea also reduced HAD considerably, possibly inhibiting at a post-replication stage during virus maturation (Fil et al, 1974).

Actinomycin D is thought to interfere with viral RNA synthesis but a specific antiviral target has not been conclusively identified (Metz and Esteban, 1972). In these experiments, at concentrations greater than 0.5 μg ml^{-1}, the drug was cytotoxic. At 0.5 μg ml^{-1} and higher concentrations, viral replication was not completely inhibited, which may account for the relatively higher HAD. Rifampicin, an inhibitor of vaccinia virus maturation (Subak-Sharpe, Timbury and Williams, 1969; Follett and Pennington, 1973) also reduced HAD, but uninfected cells also appeared to non-specifically

Table 9 The effect of metabolic inhibitors on haemadsorption to vaccinia-infected cells

Inhibitor	Concentration ($\mu g\ ml^{-1}$)	Haemadsorption (%)*		(%)†
		Infected cells	Uninfected cells	Mature virus formation
None	-	100	6, 4, 4	100
Rifampicin	100	15, 17, 16	12, 12, 11	3.2
Cytosine arabinoside	25	5, 5, 7	5, 5, 4	8.0
Hydroxyurea	38	7, 7, 11	2, 2, 4	6.0
Actinomycin D	0.5	27, 33, 41	4, 10, 8	10.0
Cycloheximide	20	9, 6, 8	6, 7, 6	7.1

*Results of triplicate assays expressed as percentages of HAD in the absence of inhibitors.

†Results expressed as average percentage infectious virus formation compared to untreated virus-infected monolayers.

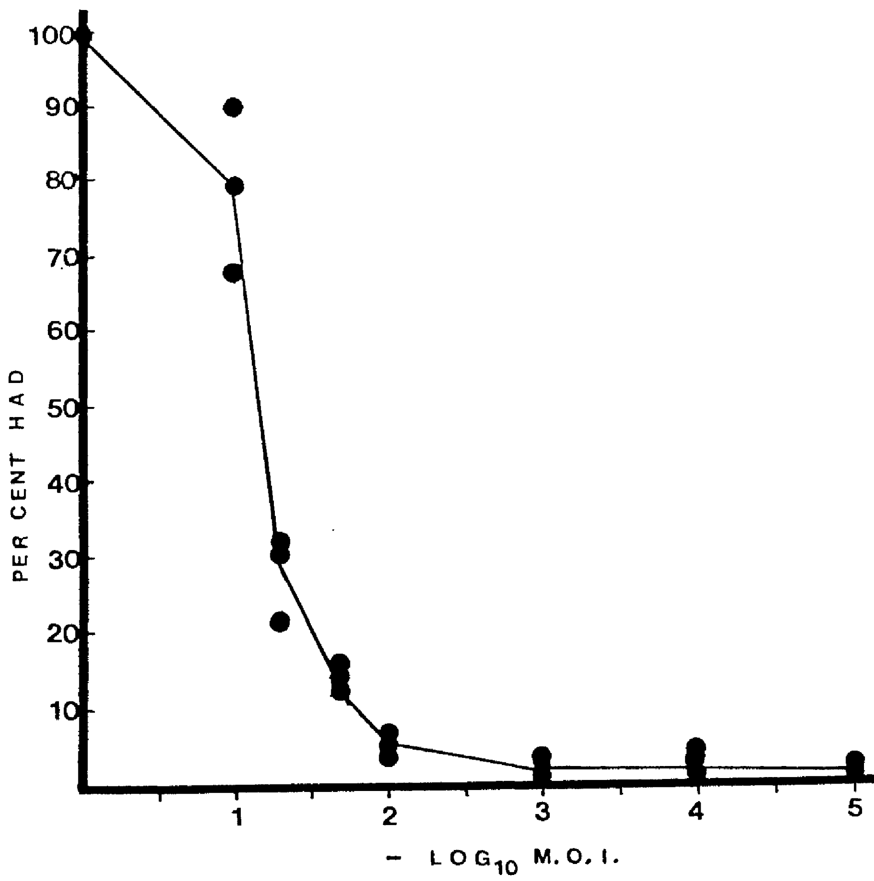
Figure 3 The effect of multiplicity of infection on cell surface VHA formation

Monolayer cultures of HeLa cells were infected with vaccinia virus at an m.o.i. of between 1 and 10^{-5} p.f.u./cell and after 24 h, monolayers were assayed in triplicate for HAD. The 100 per cent level in this experiment was taken as the average reading when an m.o.i. of 1 was employed.

Key:-

●–● haemadsorption to vaccinia-infected cells

compared to HAD to cells infected at a m.o.i. of 1 p.f.u. $cell^{-1}$ as 100 per cent.


100
90
80
70
60
50
40
30
20
10
PER CENT HAD
1
2
3
4
5
- LOG10 M.O.I.

haemadsorb slightly after rifampicin treatment. The lack of VHA production in the presence of inhibitors of viral DNA synthesis indicated that the appearance of VHA was a "late" function possibly related to virus maturation.

(iv) The effect of pH on haemadsorption to vaccinia-infected cells

The influence of pH on the binding of red blood cells to vaccinia-infected monolayers was investigated.

The results (Table 10) indicated little pH dependence for red cell binding within the range tested. Only at the extremes of the range was binding significantly depressed. Haemadsorption was optimal within the range pH 7.1 to pH 7.6 affirming the use of Dulbecco's PBS (pH 7.4) as suspending buffer in routine HAD assays. In the case of uninfected cells, no enhancement or reduction of binding was evident over the pH range tested.

(v) The effect of incubation temperature on haemadsorption of turkey red blood cells to vaccinia-infected cells

Over the temperature range tested (Figure 4) binding was maximal at 37°C and minimal at 7°C and below. At 58°C binding was still firm implying a heat stable interaction. Some non-specific adherence of erythrocytes to uninfected cells was also evident at this temperature.

(vi) The range of erythrocytes susceptible to haemadsorption to vaccinia-infected cells

Erythrocytes from eleven species were tested by quantitative

Table 10 The effect of pH on the haemadsorption of turkey red blood cells to vaccinia-infected cells

A series of phosphate-buffered saline solutions (0.85 per cent NaCl) was prepared to produce a range of pH values from 5.75 to 7.90 and two glycine-NaOH buffers were prepared for the more alkaline pH range. Vaccinia-infected HeLa cell monolayers (24 h p.i.) were washed and 5 per cent suspensions of turkey erythrocytes in buffers of varying pH added to cultures in triplicate.

Buffer pH	Haemadsorption (%)[1]
5.75	64, 67, 61
6.05	87, 68, 80
6.45	85, 90, 85
7.10	92, 74, 117
7.40	100
7.60	78, 104, 114
7.90	74, 83, 90
8.30[2]	74, 69, 70
8.70[2]	64, 63, 65

1. Compared to pH 7.4.
2. Glycine/NaOH.

Figure 4 The effect of incubation temperature on haemadsorption of turkey red blood cells to vaccinia-infected cells *

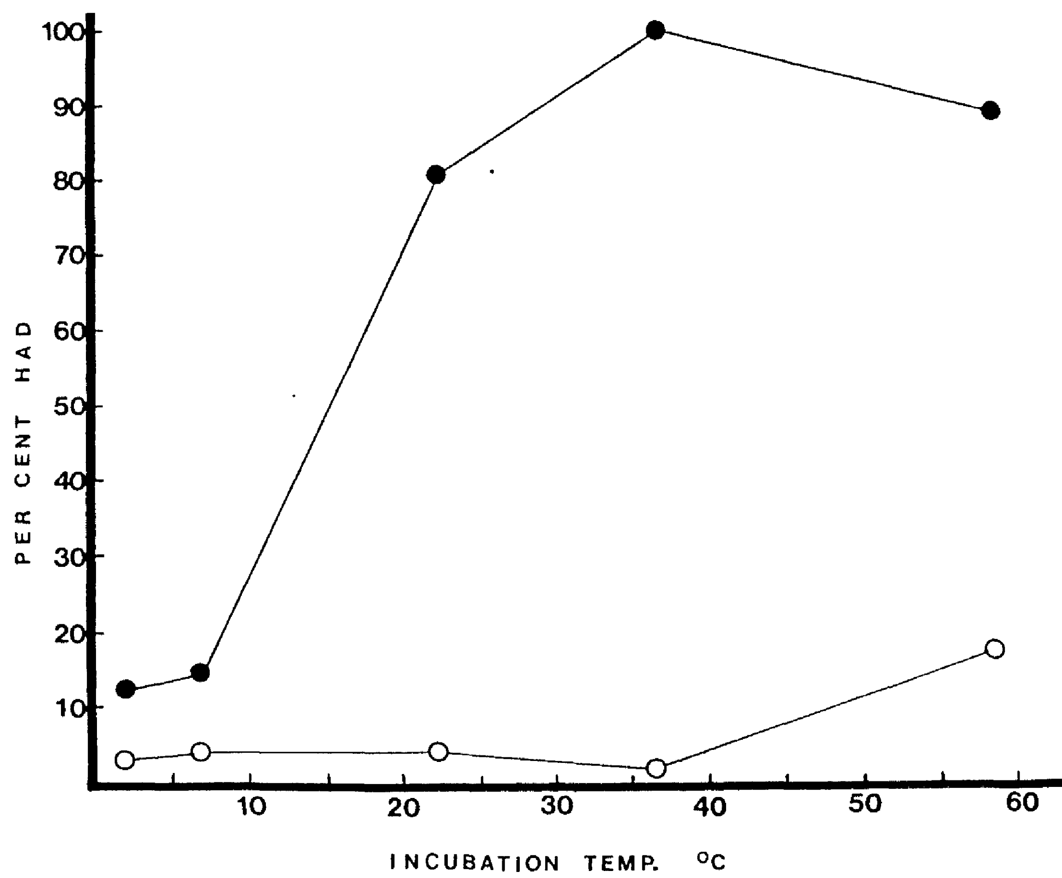
Washed 24 h virus-infected monolayers were pre-incubated at the test temperatures for 10 min, a pre-incubated turkey red cell suspension (5 per cent in PBS) was added and allowed to react for 30 min at the required temperature.

Key:-

●-● haemadsorption to vaccinia-infected cells

O-O haemadsorption to uninfected cells

* compared to HAD at 37°C to vaccinia-infected cells



100
90
80
70
60
50
40
30
20
10
PER CENT HAD
10
20
30
40
50
60
INCUBATION TEMP. °C

haemadsorption; all were suspended in PBS to 5 per cent and the assay carried out in the same way. In the case of cod erythrocytes, the incubation temperature was 22°C, since appreciable haemolysis occurred at 37°C.

In order to calculate percentage HAD, the values of E_{410}^{1cm} / E_{280}^{1cm} were determined for each red cell species by lysis in saponin/PBS, and these values substituted in the QHAD equation (Appendix 2). Apart from turkey erythrocytes significant HAD occurred (Table 11) with three red blood cell types - rabbit, African green monkey and mouse. Two other red cell types, cod and rat, adsorbed slightly and all other species were negative. That the HAD was virus-induced in all cases was shown by the lack of binding to uninfected cells.

(vii) Haemadsorption of turkey red blood cells to various cell lines infected with vaccinia virus

Seven tissue culture cell lines were infected with rabbit dermis passaged vaccinia (1 p.f.u./cell) and examined 24 hours later for HAD and intracellular virus titre. HeLa cells, despite producing the lowest yield of progeny virus, yielded the highest HAD percentage (Table 12). Virus growth was three times as efficient in BHK 21-C13 cells, but 40 per cent less efficient in terms of HAD. In HEp-2 cells little HAD occurred although viral growth was unimpaired, and this was apparent to a greater extent in the mouse epithelial cell line L-929, where HAD was virtually absent although the yields of infectious virus were similar to those obtained with HeLa cells. The results illustrate the varying susceptibility of cells to both

Table 11 The range of erythrocytes susceptible to haemadsorption to vaccinia-infected cells

	Haemadsorption (%)*	
Erythrocyte species	Infected cells	Uninfected cells
Turkey	100	5, 3, 4
African Green Monkey	52, 77, 66	3, 2, 3
Rabbit	48, 37, 46	4, 4, 5
Mouse	34, 41, 34	5, 5, 4
Rat	17, 16, 15	2, 2, 2
Cod	15, 18, 23	6, 8, 6
Human 'O'	7, 6, 10	5, 7, 6
Horse	6, 5, 7	8, 8, 6
Sheep	8, 10, 9	7, 9, 7
Cat	5, 6, 8	5, 4, 6
Dog	8, 3, 6	3, 2, 2
Chicken	4, 4, 3	3, 2, 2

* The results of three separate experiments are shown and are compared to turkey erythrocytes (100 per cent).

Table 12 Haemadsorption of turkey erythrocytes by various vaccinia-infected cell cultures

Cell culture	Haemadsorption (%)[1]		Virus yield[1]
	Infected	Uninfected	(%)
HeLa	100	5, 6, 5	100
L-132	50, 51, 49	8, 5, 7	125
BHK 21-C13	66, 67, 67	8, 7, 5	312
HEp-2	21, 21, 23	3, 3, 4	242
L-929	9, 8, 10	3, 3, 3	100
B-S-C-1	110, 94, 96	6, 4, 9	137
RK13	39, 31, 33	9, 4, 5	175

[1] Compared to vaccinia-infected HeLa cells.

propagation of infectious virus and production of VHA, and also the lack of correlation between VHA synthesis (measured by HAD) and virus growth yields.

To determine whether an inhibitor of HAD was present in L-929 cells, soluble antigen preparations were made from uninfected HeLa and L-929 cells. Turkey red cells were suspended to 5 per cent in the extracts before reaction with 24 h vaccinia-infected HeLa cell cultures and the results compared with those obtained using erythrocytes suspended in PBS. No inhibition of red cell binding was found in either HeLa or L-929 soluble extracts. In addition, the intracellular HA_{50} titre of virus-infected L-929 cell extracts was considerably reduced compared to infected HeLa cell extracts.

(viii) The effect of trypsin on haemadsorption to vaccinia-infected cells

(a) Treatment of cell monolayers prior to assay

Twenty four hours after infection of HeLa cell monolayers the overlay medium was replaced, after washing, with 1 ml of dilutions of trypsin in SFM. After 60 min at $37^{\circ}C$ plates were washed and monolayers tested for HAD. Residual trypsin activity was blocked by the addition of soy-bean inhibitor at a concentration equivalent to the original trypsin. Preliminary experiments indicated that trypsin enhanced red cell binding; cells were therefore infected at a lower multiplicity than normal, such that enhancement could be more easily detected against a low background HAD level.

Trypsin at 0.1 to 10 $\mu g\ ml^{-1}$ enhanced red cell binding to infected cells (Table 13a). Despite some destruction of the infected cell monolayer by trypsin at 10 $\mu g\ ml^{-1}$, the remaining cells were capable of binding almost three times more erythrocytes than when trypsin was absent. Below 10 $\mu g\ ml^{-1}$, trypsin did not remove cells from the monolayer but still increased HAD above normal levels.

Uninfected cells were minimally affected by trypsin at 1.0 $\mu g\ ml^{-1}$ and red cell binding was only slightly elevated. However at 10 $\mu g\ ml^{-1}$, and in the absence of gross cytotoxic effects, trypsin rendered uninfected cells almost twice as susceptible to HAD, possibly by gross cell surface protein and lipid rearrangements.

(b) Inclusion of trypsin in overlay medium during virus infection

The cells were infected at low m.o.i. to facilitate measurement of trypsin-enhanced HAD against a low background of red cell binding. After virus adsorption, cell monolayers were washed and an overlay medium containing various concentrations of trypsin applied. After 24 hours the trypsin overlay was removed and residual activity abolished by the addition of soy bean inhibitor at an equivalent concentration before assaying for HAD.

At all concentrations of trypsin tested, HAD to infected cells was enhanced (Table 13b). Trypsin concentrations of 62.5 and 125 $\mu g\ ml^{-1}$ removed cells from the monolayer but the degree of red cell binding to the remainder, was similar to that found with lower trypsin concentrations. With uninfected cells, HAD increased from the base level of 2 to 8 per cent to approximately 30 per cent in the

Table 13 The effect of trypsin on haemadsorption of turkey red blood cells to vaccinia-infected cells:-

(a) Treatment of cell monolayers prior to assay

Trypsin concentration ($\mu g\ ml^{-1}$)	Haemadsorption (%)*	
	Infected cells	Uninfected cells
0	100	7, 5, 9
0.1	150, 151, 182	14, 9, 11
0.5	173, 194, 183	n.t.
1.0	141, 143, 142	16, 9, 13
10.0	359, 382, 324	158, 195, 216

(b) Inclusion of trypsin in overlay medium during virus infection

Trypsin concentration ($\mu g\ ml^{-1}$)	Haemadsorption (%)*	
	Infected cells	Uninfected cells
0	100	7, 5, 6
7.8	189, 233, 201	n.t.
15.6	188, 220, 202	n.t.
31.2	148, 223, 185	n.t.
62.5	170, 274, 259	n.t.
125.0	247, 252, 237	39, 28, 29

n.t. = not tested

* = compared to infected cells in the absence of trypsin

presence of 125 μg ml^{-1} trypsin, although some cell removal from the monolayer was apparent at this concentration.

(ix) The effect of glutaraldehyde on the VHA-turkey red blood cell interaction

To obtain information on the chemical nature of the binding sites on both vaccinia-infected HeLa cells and turkey erythrocytes, these cells were individually treated with glutaraldehyde, a bi-functional aldehyde known to cross-link proteins on the cell surface (Avrameas and Ternynck, 1969).

Glutaraldehyde treatment of HeLa cell monolayers for either 1 or 20 min with 0.25 per cent glutaraldehyde in PBS at 23°C adversely affected the integrity of the monolayer making quantitative HAD measurements impossible. Microscopical examination showed that glutaraldehyde considerably reduced HAD, particularly on prolonged fixation.

The capacity of turkey red cells fixed with 0.25 per cent glutaraldehyde in PBS to bind to vaccinia-infected tissue culture cells was impaired and binding was reduced to base level as assessed both by QHAD assay and by microscopy.

(x) The effect of antiviral antibody on haemadsorption of turkey red blood cells to vaccinia-infected cells

To investigate the specificity of the erythrocyte/infected cell interaction, the capacity of rabbit antiVACγG to block HAD was investigated. Normal rabbit γ-globulin (NγG) of similar protein concentration in PBS was employed in control assays.

(a) The inhibition of haemadsorption to vaccinia-infected cells by specific antiviral antibody

Taking the haemadsorption-inhibition ($HADI_{50}$) assay end point as that dilution of serum which reduced HAD to 50 per cent of the control (PBS) level, the antiVACγG had an $HADI_{50}$ titre of 1/100 (Figure 5), and abolished red cell binding completely at a dilution of 1/20. When NγG concentrate replaced antiVACγG, no inhibition of HAD occurred at dilutions of 1/5 or greater.

The haemagglutination-inhibition (HAI_{50}) titre of the antiVACγG concentrate was also tested, the highest dilution of serum giving 50 per cent inhibition of agglutination, was 1/16,000.

(b) Blocking of the haemadsorption-inhibition assay by a vaccinia soluble antigen preparation

Antigen dilutions as high as 1/1024 exhibited some anti-HAD activity (Table 14). The blocking titre of the soluble antigen preparation, i.e. that which reduced HAD by 50 per cent, was 1/8 - 1/4.

(c) The resumption of haemadsorption to vaccinia-infected cells after blocking by antiviral antibody

In preceding experiments, antibody to vaccinia efficiently blocked HAD to infected cells. Two further experiments were carried out to determine whether, on removal of antibody, the haemadsorbing antigen reappeared at the cell surface.

In the first experiment, monolayers were infected employing

Figure 5 The effect of antiviral antibody on haemadsorption of turkey red blood cells to vaccinia-infected cells *

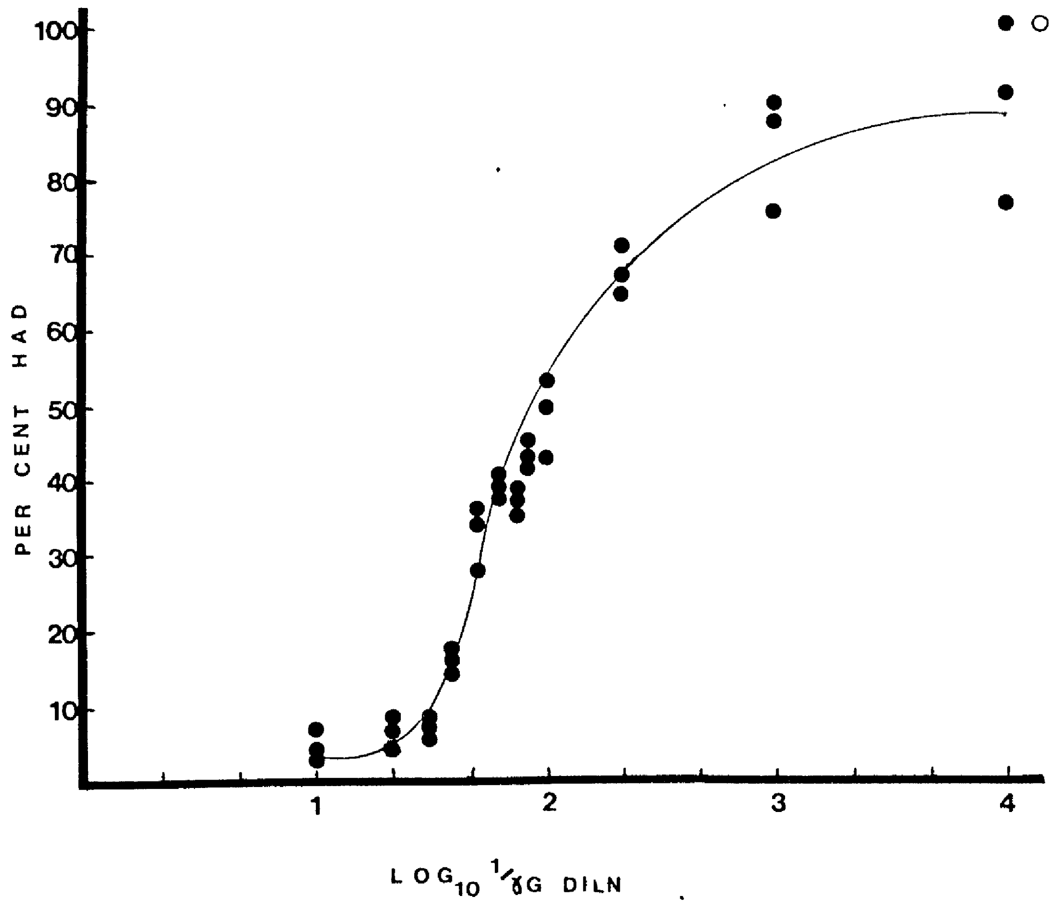
Serial dilutions of antiVACγG were applied in 0.75 ml volumes to washed, 24 h virus-infected monolayers and after incubation (30 min, $37^{o}C$), the cell sheet was washed lightly with PBS before testing for HAD.

Key:-

●—● + antiVACγG

○ + NγG

* compared to HAD to vaccinia-infected cells treated with NγG.



100
90
80
70
60
50
40
30
20
10
PER CENT HAD
1
2
3
4
LOG10 1/γG DILN

Table 14 Blocking of the haemadsorption-inhibition assay by a vaccinia soluble antigen preparation

A soluble antigen preparation from vaccinia-infected HeLa cells was serially diluted in PBS and mixed with equal volumes of 1/15 anti-VACγG. After incubation at 37°C for 30 min to allow antigen/antibody interactions, the mixtures were tested for their ability to reduce HAD; the dilution of antiserum employed (1/30 final dilution) was, from the previous experiment, sufficient to block HAD.

Dilution of cell extract	Anti VACγG dilution	Haemadsorption (%)[1]
-	(NγG)	100
1/2	1/15	60, 56, 52
1/4	1/15	55, 69, 72
1/16	1/15	45, 45, 51
1/32	1/15	32, 31, 37
1/64	1/15	31, 33, 36
1/256	1/15	13, 16, 20
1/1024	1/15	10, 15, 12
PBS	1/15	4, 3, 4
uninfected cells	-	4, 6, 4

[1]Compared to infected cells incubated with normal rabbit γ-globulin

the normal virus adsorption period. After washing, the cell monolayers were overlaid with 1.0 ml per plate of calf serum-free medium (CSFM) containing rabbit antiVACγG or rabbit NγG (1.5 per cent v/v). After a further 24 h at 37°C overlays were replaced with CSFM. At various times thereafter the plates were tested for HAD (Table 15a). After removal of anti-viral γG red cell binding increased slowly, and in the 24 hours after removal of the overlay, increased by the same amount as the control (NγG treated) cells. Plaque titration of infectious virus 24 h after removal of antiserum (48 h p.i.) yielded only 40 per cent of the virus found in untreated plates (48 h p.i.), whereas in the case of plates overlaid with antiserum throughout the 48 h period the infectious virus titre was only 8 per cent of the control.

The second experiment was designed to measure the rate of reappearance of VHA at the cell surface of 24 h vaccinia-infected cells after brief treatment with antiviral serum. The temporal reappearance of VHA was first measured. Haemadsorption increased slowly as soon as antiVACγG was removed (Table 15b) and rose to 50 per cent of the control level by 4.5 h. At this stage VHA was just detectable intracellularly but at a level well below control.

To determine whether protein synthesis was necessary for reappearance of VHA, cycloheximide was incorporated in the overlay medium after removal of antiVACγG. In this case by 5 h after antibody treatment, HAD increased from background to approximately 75 per cent of the control level when no inhibitor was present (Table 15c). During the 5 hour period, HAD to control (NγG) infected cells increased

Table 15(a) The resumption of haemadsorption to vaccinia-infected cells after blocking by anti-viral antibody: cells incubated with antibody during the virus growth cycle (24 h)

Transpose to table 15(b)

Washed 24 h vaccinia-infected cells were treated with CSFM containing anti-VACγG or NγG (5% v/v) for 30 min at 37°C. After washing (except for control plates) monolayers were overlaid with CSFM and periodically assayed for HAD and 'soluble' HA_{50} titre.

Rabbit γG contained in overlay medium (1.5% v/v)	Time after removal of overlay (h)[1]	% Haemadsorption[2]	
		Infected cells	uninfected cells
NγG	0	100	3, 4, 2
anti-VACγG	0	21, 17, 17	2, 2, 2
anti-VACγG	0.3	13, 10, 12	n.t.
anti-VACγG	0.6	12, 14, 11	n.t.
anti-VACγG	1.0	15, 13, 13	n.t.
anti-VACγG	2.0	14, 16, 17	n.t.
anti-VACγG	3.5	17, 17, 20	n.t.
anti-VACγG	5.5	20, 21, 23	n.t.
anti-VACγG	24	31, 29, 33	3, 4, 3
anti-VACγG	not removed	11, 11, 13	4, 3, 2
NγG	24	129, 112, 119	n.t.

[1] The antiserum overlay was removed at 24 h p.i. (= zero time)

[2] Compared to haemadsorption by 24 h infected cells incubated in normal rabbit γG overlay medium

n.t. = not tested.

Table 15(b) The resumption of haemadsorption to vaccinia-infected cells after blocking by antiviral antibody: cells incubated with antibody after the virus growth cycle

Rabbit γG contained in overlay medium (5% v/v)	Time after removal of overlay (h)[1]	% Haemadsorption[2] Infected cells	uninfected cells	VHA_{50}[3] titre
NγG	0	100	4, 3, 4	16
anti-VACγG	0	9, 8, 9	3, 3, 4	0
anti-VACγG	0.5	13, 12, 11	n.t.	0
anti-VACγG	1.0	14, 15, 17	n.t.	0
anti-VACγG	1.5	26, 22, 22	n.t.	0
anti-VACγG	2.5	41, 41, 43	n.t.	0
anti-VACγG	4.5	47, 51, 50	5, 4, 4	1
NγG	4.5	103, 100, 105	3, 3, 2	16
anti-VACγG	not removed	6, 6, 7	3, 3, 2	0

[1]The antiserum overlay was applied 24 h p.i. and removed after 30 min (= zero time)

[2]Compared to haemadsorption by 24 h infected cells treated with normal γG

[3]Titre of 'intracellular' haemagglutinin in infected cultures

n.t. = not tested

Table 15(c) Resumption of haemadsorption to vaccinia-infected cells after blocking by antiviral antibody: the effect of cycloheximide

Virus-infected cells (24 h p.i.) were washed and overlaid with either rabbit NγG or rabbit anti-VACγG for 30 min at 37°C. After washing, the cell monolayers were overlaid with 1.0 ml of SFM with or without cycloheximide at 20 μg ml^{-1}. Five hours later the plates were assayed for HAD.

First overlay medium (rabbit γG, 5% v/v)	Second overlay medium	Time after replacement of first overlay medium (h)	% Haemadsorption[2]	
			infected cells	uninfected cells
NγG	SFM[1]	0	100	8, 8, 7
anti-VACγG	SFM	0	11, 8, 10	9, 7, 8
anti-VACγG	SFM	5	77, 73, 76	7, 5, 8
anti-VACγG	SFM + anti-VACγG	5	12, 11, 10	9, 7, 7
NγG	SFM + NγG	5	126, 124, 123	7, 9, 8
anti-VACγG	SFM + cycloheximide	5	28, 29, 24	10, 6, 6
NγG	SFM + cycloheximide	5	92, 90, 97	6, 6, 9

[1] Eagle's medium without calf serum

[2] Compared to vaccinia-infected cells treated only with NγG and SFM

by approximately 25 per cent but in cells treated with antiVACγG, the level increased from background to 75 per cent of the control level. Cycloheximide totally suppressed the increase in HAD to control cells and markedly reduced the increase in HAD to antiVACγG treated cells.

(d) The effect of antiviral antibody on turkey red blood cells haemadsorbed to vaccinia-infected cells

Anti-vaccinia serum has been shown to remove VHA from haemagglutinated red blood cells (Burnet and Stone, 1946) and the "regenerated" cells could be re-agglutinated by VHA preparations. An experiment was designed to ascertain whether this effect could also be produced on red blood cells bound to vaccinia-infected cells by the addition of antiviral serum after HAD had taken place.

The progressive elution of adsorbed red cells by specific antiserum is shown in Figure 6. Elution was apparent soon after the addition of antibody and after 45 minutes incubation, red cell binding was reduced by 60 per cent although NγG caused no elution of bound erythrocytes. Passive elution of bound red cells was not apparent.

(xi) The purification of vaccinia haemagglutinin

An attempt was made to purify VHA from vaccinia-infected HeLa cells by the 2 stage density gradient centrifugation method of Gurvin and Haukenes (1973). Uninfected cells and cells infected in the presence of an inhibitor of viral DNA synthesis were subjected to the same purification procedure. The results of the purification

Figure 6 The effect of antiviral antibody on turkey red blood cells haemadsorbed to vaccinia-infected cells ✳

Virus-infected cultures were tested for HAD at 24 h p.i. by the addition of a 5 per cent suspension of turkey red cells. After 30 min incubation at $37^{o}C$, the excess red cells were removed by washing. The cells were then overlaid with either NγG or anti-VACγG at a dilution of 1/10 in PBS (0.75 ml per plate). At various times after, the plates were washed with PBS to remove unattached red cells and the bound red cells estimated by QHAD.

Key:-

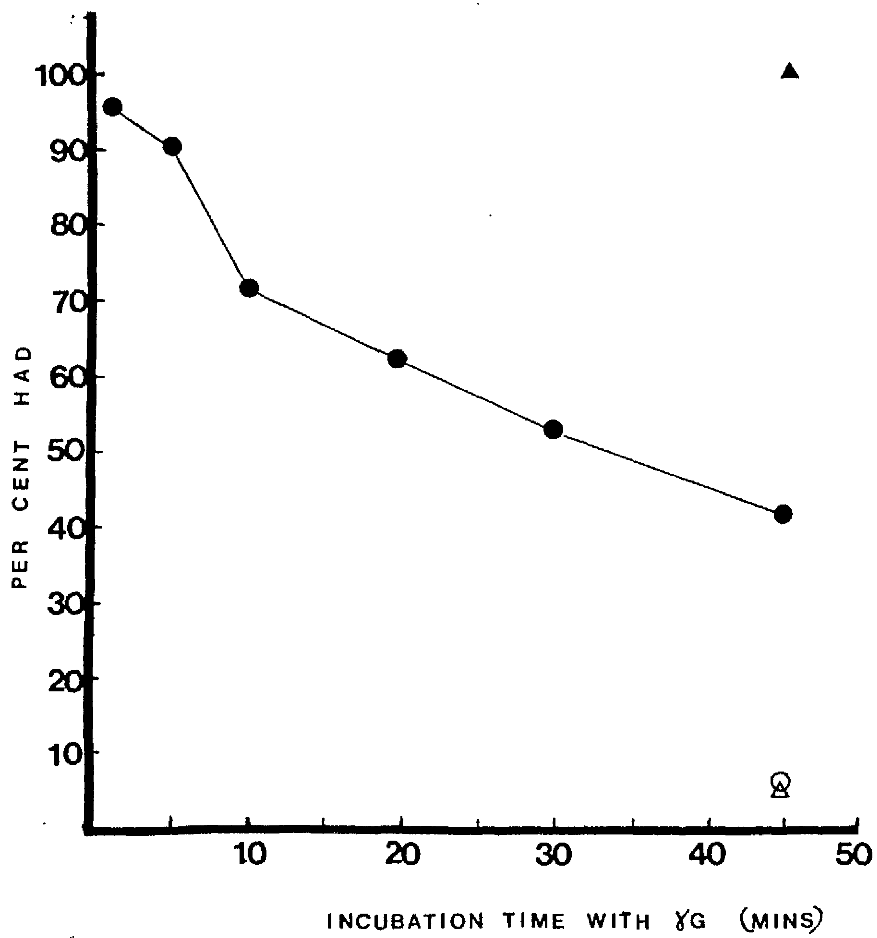
●—● vaccinia-infected cells + antiVACγG

○ uninfected cells + antiVACγG

▲ vaccinia-infected cells + NγG

△ uninfected cells + NγG

✳ compared to HAD to vaccinia-infected cells treated with NγG.



PER CENT HAD
100
90
80
70
60
50
40
30
20
10
10
20
30
40
50
INCUBATION TIME WITH γG (MINS)

procedure are shown in Figure 7 and Table 16.

The material extracted from vaccinia-infected untreated cells had an HA titre of 1/1024 but uninfected and ara-C-treated infected cells were both devoid of HA activity; virus replication was inhibited by 99.8 per cent in ara-C-treated cells. The distribution of protein over the gradients was similar in all three cases and the peak of HA activity from extracts of infected cells was recovered in three major fractions ranging in density from 1.09 to 1.14 gm l^{-1} (Figure 7a). The average activity in terms of HA_{50} units per μg protein was lower than in the original material (Table 16). The VHA peak did not coincide with any major protein peak.

The three major fractions from each of the gradients were individually pooled and subjected to Stage 2 purification. The protein distributions in this case showed a marked difference between the "infected" preparation and the remaining two preparations, both of which displayed similar distributions (Figure 7b, 7c). In the latter cases the bulk of the protein was concentrated towards the bottom of the gradients although the top fractions were also rich in protein. The protein from the "infected" preparation steadily increased in quantity towards the top of the gradient and the protein peak coincided with the one peak of HA activity found in the density gradient in the top fraction. In terms of specific activity this represented a 10-fold purification of VHA from Stage 1 to Stage 2 (Table 16). This semi-purified material was subjected to SDS-polyacrylamide gel electrophoresis. The complex banding pattern produced (Plate 4) suggested that this semi-purified material represented a heterogeneous mixture of cellular and viral polypeptides.

Figure 7 The purification of vaccinia haemagglutinin:- comparison of sucrose density gradient fractions from uninfected HeLa cells and cells infected in the presence or absence of cytosine arabinoside

Extracts of i) 24 h-vaccinia-infected cells (Figure 7a)

ii) 24 h-vaccinia-infected cells in the presence of ara-C (Figure 7b)

iii) uninfected cells (Figure 7c)

were subjected to a two-stage sucrose density gradient purification procedure to purify VHA (Gurvin and Haukenes, 1973).After centrifugation of a "soluble" cell extract on density gradients in stage (1), the peak VHA fractions in a) were pooled, as were the corresponding fractions in b) and c), and re-centrifuged on a second, but different gradient. From this stage (2) centrifugation peak fractions of VHA activity could be demonstrated in a). For each fraction sucrose and protein concentrations were also measured.

Key:-

o—o protein ($\mu g\ ml^{-1}$)

□—□ haemagglutinin

△—△ sucrose (per cent w/v)

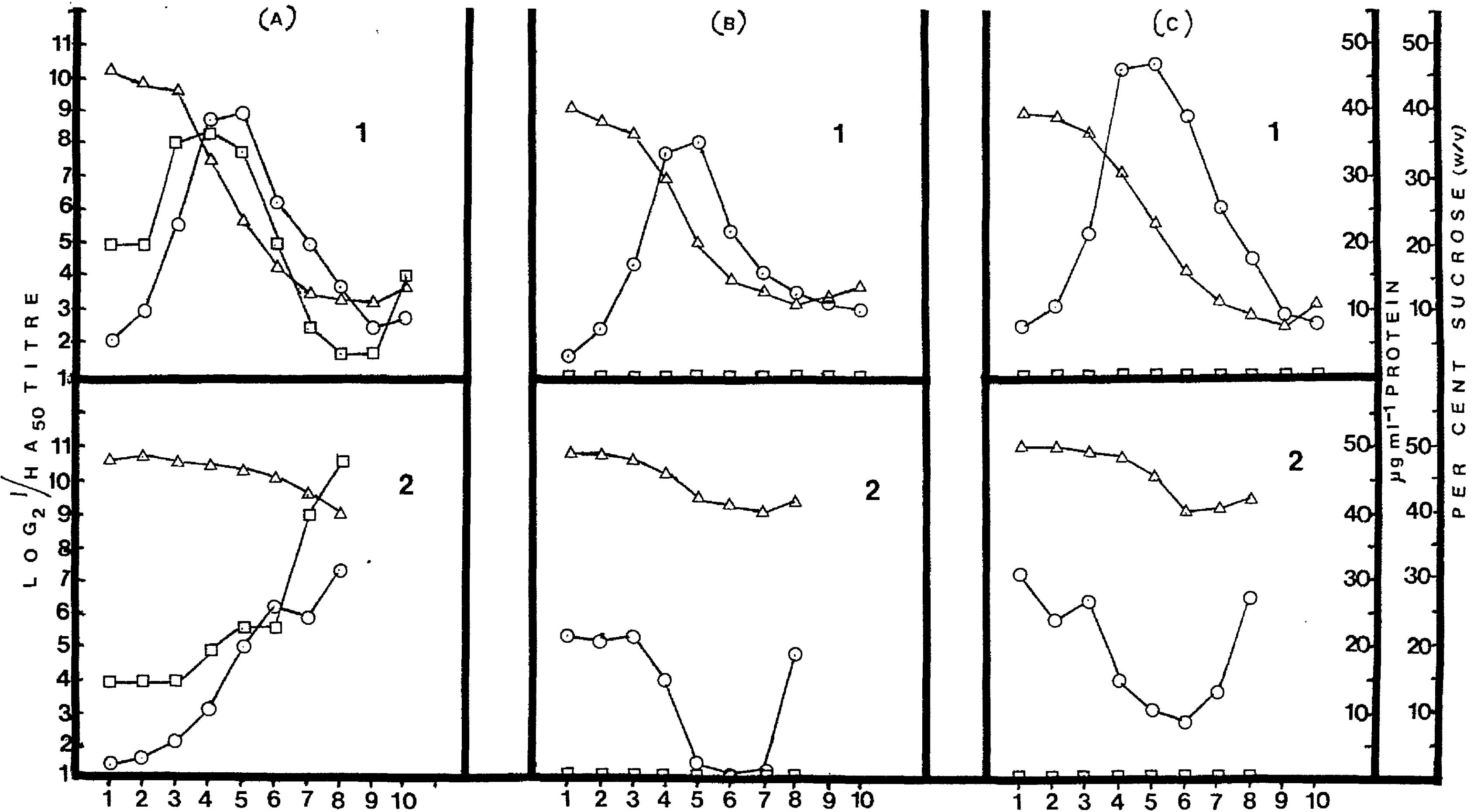
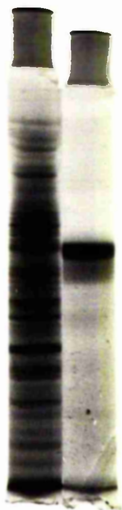

(A)
(B)
(C)
1
2
LOG2 1/HA50 TITRE
μg ml⁻¹ PROTEIN
PER CENT SUCROSE (w/v)
FRACTION NUMBER

Table 16 Purification of vaccinia haemagglutinin: analysis of sucrose density gradient fractions

Sample	Infected cells			ara-C treated infected cells		uninfected cells	
	protein mg ml^{-1}	$1/HA_{50}$ titre	HA_{50} units per μg protein	Protein mg ml^{-1}	$1/HA_{50}$ titre	protein mg ml^{-1}	$1/HA_{50}$ titre
Sonicate	2.18	1024	0.47	1.94	0	2.04	0
1000 g supernatant of sonicate	1.74	1024	0.59	1.60	0	1.88	0
sucrose density gradient I peak fraction	1.58	512	0.32	1.40	0	1.90	0
sucrose density gradient II peak fraction	1.28	4096	3.2	0.76	0	1.01	0

Plate 4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis of "purified" vaccinia haemagglutinin

VHA purified by sucrose density gradient fractionation of extracts of vaccinia-infected HeLa cells was subjected to SDS-PAGE according to the method of McNiven *et al* (1972). The resulting polypeptide pattern (left on plate) was compared to that obtained with human serum albumin (right on plate).



C. INTERACTIONS BETWEEN CONCANAVALIN A AND VACCINIA-INFECTED CELLS

A variety of plant lectins capable of binding to specific sugars have been described (reviewed by Lis and Sharon, 1973). In particular, con A, which binds to α-D-glucopyranose, α-D-mannopyranose, α-D-fructofuranose and their glycosides (Poretz and Goldstein, 1970), has been used to monitor changes in the distribution of sugars on the surface of mammalian cells (Nicholson, 1974). In the following experiments con A was used to detect surface changes in vaccinia-infected cells.

(i) Concanavalin A agglutination of vaccinia-infected human embryonic lung cells

Zarling and Tevethia (1971) and Bandlow *et al* (1973) reported that vaccinia-infected cells became agglutinable by con A as early as two hours p.i..

Here, the method used by Zarling and Tevethia was modified, and cell agglutination was assessed not by macroscopic patterns in plastic trays but by direct microscopical examination of the cells. Preliminary experiments demonstrated the susceptibility of the HeLa cell line itself to agglutination in the presence of con A at 1 mg ml^{-1}. A primary cell line, human embryonic lung cells was therefore selected. The results (Table 17) showed that preferential agglutination by con A of virus-infected cells could be demonstrated within 3 to 5 h of infection. In saline, although no agglutination was initially apparent, some auto-agglutination occurred with infected cells, but not with uninfected cells.

Table 17 Concanavalin A agglutination of suspensions of vaccinia-infected human embryonic lung cells

Time (h p.i.)	Degree of agglutination*	
	Autoagglutination	Agglutination by Con A
1	0	1
3.5	1	2
6.5	1	2
8	1	3
10	1	3
14	1	3
18	1	3
24	1	3

* Results of four assays; degree of agglutination was assessed visually,

0 = no agglutination
1 = 2-3 cells clumped
2 = 6-20 cells clumped
3 = > 20 cells clumped

Uninfected cells tested at all times showed no autoagglutination or agglutination by con A.

Clumps of autoagglutinated cells consisted of two to three cells early in infection and later increased to six or more. However it did not resemble the massive conglomerates of $>$ 100 cells produced in the presence of con A. Autoagglutinability was evident as early as 3.5 h p.i. and increased moderately thereafter. It may be related to the increased amount of cell surface mucopolysaccharide reported to be present during vaccinia infection (Bubel and Lambert, 1967) or the virus induced "fusion from without" observed by Mbuy and Bubel (1976).

(ii) Concanavalin A binding to vaccinia-infected cells

(a) Determination of optimum concanavalin A concentration for coating human red blood cells

Since uninfected HeLa cells were agglutinated by con A and presumably therefore also bound con A in significant amounts, it was of interest to determine whether vaccinia-infected cells might preferentially bind more con A during infection. In initial experiments the protocol of Bubel and Blackman was followed using human rather than mouse erythrocytes since the latter react with both con A and VHA. However, the high degree of red cell binding did not allow precise quantitation, especially of differences in binding between infected and uninfected cells due to red cell binding by excess con A.

An alternative approach, similar to that used by Furmanski et al (1972) and Branny, Sainerova and Sovova (1976) was used, whereby instead of sequential treatment of the test cell monolayer with con A and human erythrocytes, the erythrocytes were pre-coated with con A

prior to reaction with the test cells. The binding of con A to erythrocytes apparently leaves other binding sites exposed on the con A molecule which can further react with receptor sites on the surface of the test cells (Leon and Young, 1970). This method, where excess lectin was removed prior to test, provided a more sensitive assay for con A binding sites. The optimum concentration of con A to be used for red cell coating was first determined.

The comparative binding of con A-red cells to both infected and uninfected cells is shown in Figure 8. At concentrations of less than 50 $\mu g\ ml^{-1}$ the amount of con A bound to erythrocytes was insufficient to detect differential binding to virus-infected cells. Only at levels of 100 μg of con A and above did con A-red cells show selective binding properties. A level of 200 $\mu g\ ml^{-1}$ was the minimum concentration of con A for coating erythrocytes to allow enhanced adhesion to virus-infected test cells to be easily and consistently distinguished from background binding to uninfected test cells. Con A -red cells prepared with 1000 $\mu g\ ml^{-1}$ con A showed greater binding to infected cells but the increased inter-red cell binding and agglutination interfered with the monolayer binding test.

In all further experiments to determine con A binding, human red cells were treated with the lectin at 200 $\mu g\ ml^{-1}$.

(b) <u>The effect of vaccinia infection on concanavalin A binding to HeLa cells</u>

Con A-red cells prepared as described above were allowed to react with monolayers of infected or uninfected HeLa cells at various

Figure 8 Determination of the optimum concanavalin A concentration for coating human red blood cells

Aliquots of 0.5 ml packed human 'O' red blood cells were mixed with 2.5 ml volumes of Con A solutions (0 - 1000 $\mu g\ ml^{-1}$ in PBS) and incubated for 60 min at $37^{o}C$. Coated red cells (con A-red cells) were washed three times in PBS and stored at $4^{o}C$ as a 2 per cent solution in PBS prior to use. Vaccinia-infected cell monolayers (24 h p.i.) were treated with con A-red cells and the degree of binding assessed spectrophotometrically as in the QHAD test. The results were calculated using the average $E_{410\ nm}/E_{280\ nm}$ for con A-red cells taken from the measured values for red cells treated with each concentration of con A.

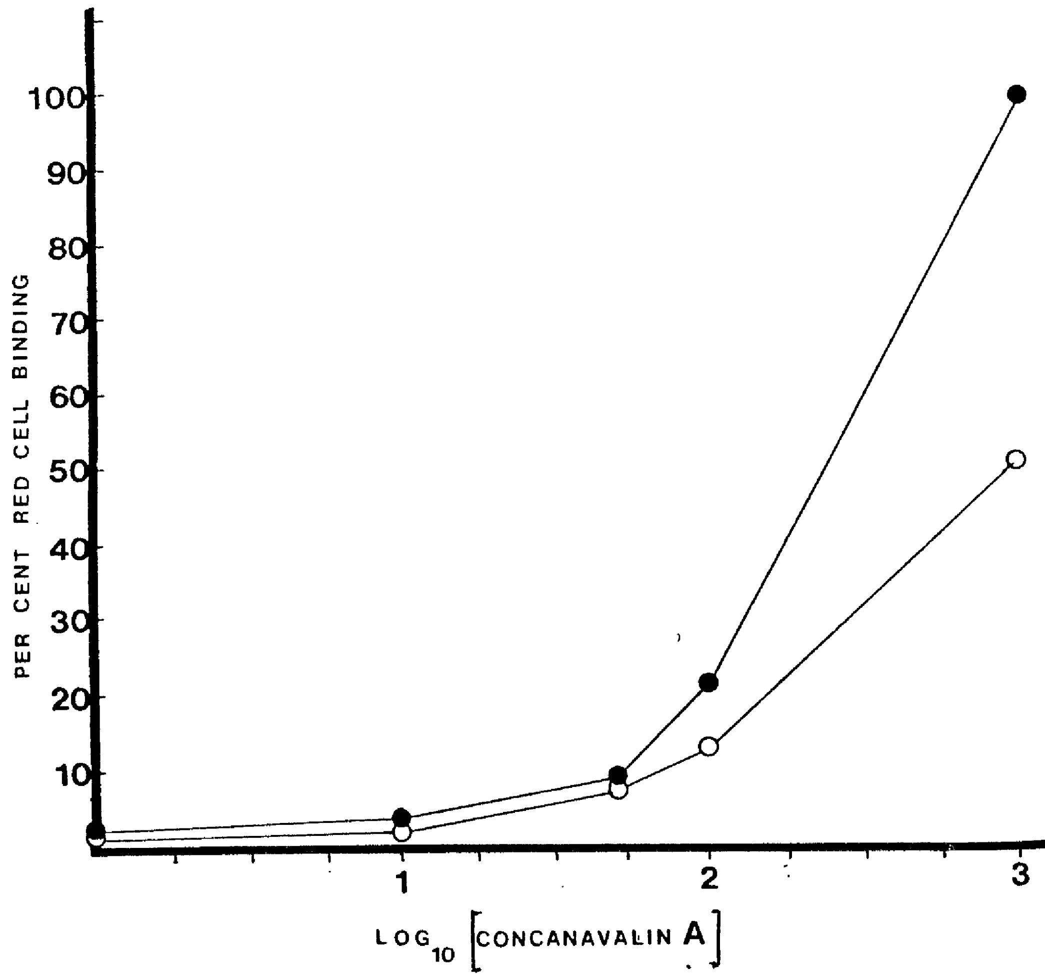
Key:-

●–● vaccinia-infected cells

○–○ uninfected cells

concanavalin A is expressed in $\mu g\ ml^{-1}$

compared to binding at a coating concentration of 1000 $\mu g\ ml^{-1}$.


PER CENT RED CELL BINDING
100
90
80
70
60
50
40
30
20
10
1
2
3
LOG10 [CONCANAVALIN A]

times after vaccinia infection to determine the degree of con A binding.

In both uninfected and vaccinia-infected HeLa cells a fluctuating pattern of con A-red cell binding emerged during the test period, particularly evident from 0 to 14 h (Figure 9). From 14 h on, binding to both was more consistent as was a reproducible, significant difference in binding between uninfected and vaccinia-infected cells. Fluctuations in con A-red cell binding to infected cells differed from uninfected cells, the most marked difference being a sharp reduction by one hour p.i. followed by a recovery. Consistent binding differences between uninfected and vaccinia-infected cells became clear by 14 h p.i. This was not related to the appearance of cell surface VHA; human "O" red cells were not haemadsorbed to vaccinia-infected cells and the adsorption of con A-red cells was not inhibited by anti-vaccinia serum.

(c) The effect of metabolic inhibitors on concanavalin A binding to uninfected and vaccinia-infected HeLa cells

This experiment was carried out employing the same conditions used to investigate the effects of inhibitors on HAD.

Treatment of cells with each of the inhibitors had a profound effect on the red cell binding (Table 18). In all cases the binding to virus infected monolayers was greater than to monolayers infected in the absence of inhibitor, and increased four-fold in the presence of cycloheximide. The inhibition of cellular metabolism apparently led to

Figure 9 The effect of vaccinia infection on concanavalin A binding to HeLa cells

Uninfected and vaccinia-infected HeLa cell monolayers were tested at various times after infection for the ability to bind con A-coated human 'O' erythrocytes by incubation at 37°C, washing and removal of "free" red cells, and determination of binding by spectrophotometry after lysis of the erythrocytes.

Key:-

●—● vaccinia-infected cells

○—○ uninfected cells

compared to binding to vaccinia-infected cells at 24 h p.i.

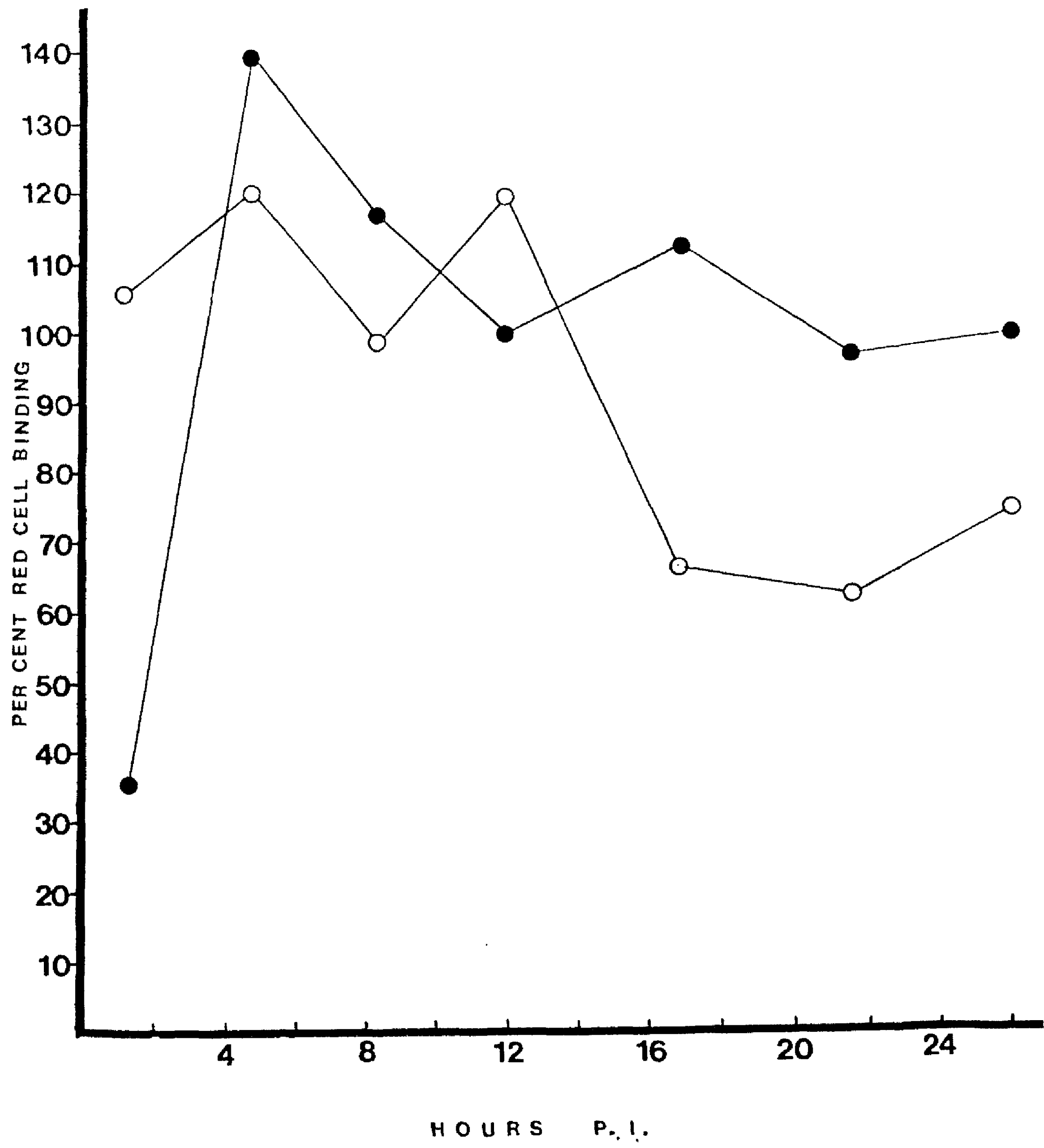

140
130
120
110
100
90
80
70
60
50
40
30
20
10
PER CENT RED CELL BINDING
4
8
12
16
20
24
HOURS P. I.

Table 18 The effect of metabolic inhibitors on concanavalin A binding to uninfected and vaccinia-infected HeLa cells

After the virus adsorption period, cells were overlaid with SFM containing either rifampicin, ara-C, cycloheximide or actinomycin D and incubated at 37°C before assaying for con-A binding sites

Inhibitor	Concentration ($\mu g\ ml^{-1}$)	%Con A-red cell binding*	
		Infected cells	Uninfected cells
None	-	100	66, 53, 66
Rifampicin	100	116, 114, 114	77, 68, 75
Cytosine arabinoside	25	151, 132, 149	43, 50, 38
Cycloheximide	20	420, 300, 394	277, 205, 340
Actinomycin D	0.5	204, 189, 214	253, 239, 213

*Compared to cells infected with vaccinia in the absence of inhibitors.

an increase in the number of con A binding sites at the cell surface, since enhanced binding was also demonstrable when uninfected cells were incubated with medium containing inhibitor. The presence of inhibitors did not affect the binding of untreated human erythrocytes. The greatest increase in con A-red cell binding occurred with cycloheximide and actinomycin D, potent inhibitors of protein synthesis and RNA synthesis respectively. Cytosine arabinoside and rifampicin inhibit predominantly viral rather than cellular functions which may partly explain the lesser effect of these inhibitors on con A binding to uninfected cells. They did however enhance binding to virus-infected cells but to a lesser degree than the other inhibitors tested. Progeny virus yield was inhibited to the levels previously found (page **85**).

(d) Differentiation between the binding sites on vaccinia-infected cells for turkey erythrocytes and concanavalin A-coated human erythrocytes

Human red cells were not spontaneously haemadsorbed to vaccinia-infected HeLa cells, but if pre-coated with con A they did adhere to virus-infected cells, suggesting that the second haemadsorption reaction was not mediated by VHA but was attributable to receptor sites on the cell surface with an affinity for con A. As shown above, these receptors are present also on uninfected HeLa cells.

In this experiment the relationship between the two red cell binding specificities was investigated, to determine whether prior binding of one red cell type interfered with subsequent binding of the other erythrocyte. The results are presented in Table 19.

Table 19 Differentiation between the binding sites on vaccinia-infected cells for turkey erythrocytes and concanavalin A-coated human erythrocytes

Virus-infected monolayer cultures (24 h p.i.) were overlaid first with either PBS, rabbit antiVACγG, con A-red cells or turkey red blood cells. After 30 min at 37°C the overlay was replaced by a second and occasionally third overlay and those were removed after 30 min at 37°C. The monolayers were then examined by phase contrast microscopy for erythrocyte binding. Human and turkey erythrocytes were easily distinguished microscopically by differences in size and shape.

Sample	Sequential treatment of cell monolayers[1]			Binding of erythrocytes to cell monolayers[2]			
				Con-A-human erythrocytes		turkey erythrocytes	
	First	Second	Third	infected cells	uninfected cells	infected cells	uninfected cells
1	PBS	con A/human	None	++	+	–	–
2	PBS	turkey	"	–	–	+++	–
3	AVACγG	turkey	"	–	–	–	n.t.
4	AVACγG	con A/human	"	+++	n.t.	–	–
5	con-A/human	turkey	"	++	+	++	–
6	turkey	con A/human	"	++	+	+++	–
7	con-A/human	AVACγG	turkey	++	+	–	–
8	turkey	AVACγG	none	–	–	+	n.t.
9	con-A/human	AVACγG	"	++	n.t.	–	–

[1]Each treatment was for 30 min at 37°C

[2]–, no binding; +, ++ and +++ indicate increasing degrees of red cell binding.

n.t. = not tested

Concurrent binding of turkey and con A-red cells to the same virus-infected cell was observed in sample 5. Prior binding of turkey red cells had little effect on the later binding of con A-red cells (Sample 6) but con A-red cells partially masked some turkey erythrocyte binding sites (Sample 5). Anti VACγG completely blocked binding of turkey erythrocytes to infected monolayers but had no effect on the binding of con A-red cells which indeed was possibly enhanced (Samples 3 and 4). There was no inter-red cell binding between turkey erythrocytes and con A-red cells by reaction with excess available con A sites on the human red cells (Sample 5). Con A-red cells bound to either infected or uninfected monolayers were not eluted by anti-viral antibody (Samples 7 and 9) whereas turkey erythrocytes were eluted (Sample 8). Antibody however must have bound to sites not masked by the con A-red cells since further adherence of turkey erythrocytes was prevented (Samples 7 and 5).

The above findings indicated that turkey red blood cells and con A have different receptor sites on vaccinia-infected cells.

D. THE SIALIC ACID CONTENT OF VACCINIA-INFECTED CELLS

Bubel and Lambert (1967) reported that increased quantities of acidic mucopolysaccharide were found in vaccinia-infected cells, compared to uninfected cells, and sialic acid synthesis was related temporally to virus synthesis. Later, Bubel and Blackman (1975) attempted to demonstrate a cell surface location for sialic acid by treatment of vaccinia-infected cells with neuraminidase. The unchanged electrophoretic mobility suggested that the increased amount of sialic

acid was located internally and not accessible to neuraminidase.

Here, the sialic acid content of cells was compared in the absence of virus infection, 24 hours after virus infection and 24 hours after infection but with ara-C included in the medium at 25 $\mu g\ ml^{-1}$.

Three estimations of sialic acid content were each made from 4×10^7 monolayer HeLa cells. The results, presented in Table 20, suggested that no overall stimulation of sialic acid synthesis occurred in vaccinia-infected HeLa cells by 24 hours p.i. Indeed cells seemed to contain significantly less sialic acid than uninfected control cells. Cytosine arabinoside prevented the reduction in sialic acid content.

The sialic acid content of vaccinia-infected HeLa monolayers from zero to 32 h p.i. was measured. Haemadsorption and infectious virus titres were also monitored. The results (Figure 10) showed a rapid decline in sialic acid content following entry of virus into the host cell from 1.5 h p.i. to 14.0 h p.i.. During the parallel rise in virus titre and HAD from 10 h p.i. onwards, the total cell sialic acid content also increased, though only from around 15 h p.i.. By 24 h p.i., as indicated above, the sialic acid level was significantly lower than in control uninfected cultures.

E. HELA CELL PLASMA MEMBRANES : PREPARATION, PURIFICATION, AND CHARACTERISATION

(i) Preparation and purification

Increasing awareness of the importance of the cell surface

Table 20 The sialic acid content of HeLa cells 24 h after infection with vaccinia virus

	(μg per mg protein)		
Uninfected cells	4.90	4.41	4.17
Infected cells	2.98	2.75	1.83
Infected cells + cytosine arabinoside	3.61	3.35	4.64

Figure 10 The sialic acid content of vaccinia-infected cells monitored during virus growth : comparison with the appearance of intracellular virus and cell surface haemagglutinin *

Vaccinia-infected HeLa cell monolayers were examined at various stages during the virus growth cycle for the appearance of infectious virus and HAD, and these were compared with the sialic acid content of cells sampled at the same time. Whole cells were scraped from monolayers, washed and assayed for sialic acid content and also total protein, and the results expressed as μg sialic acid per mg protein.

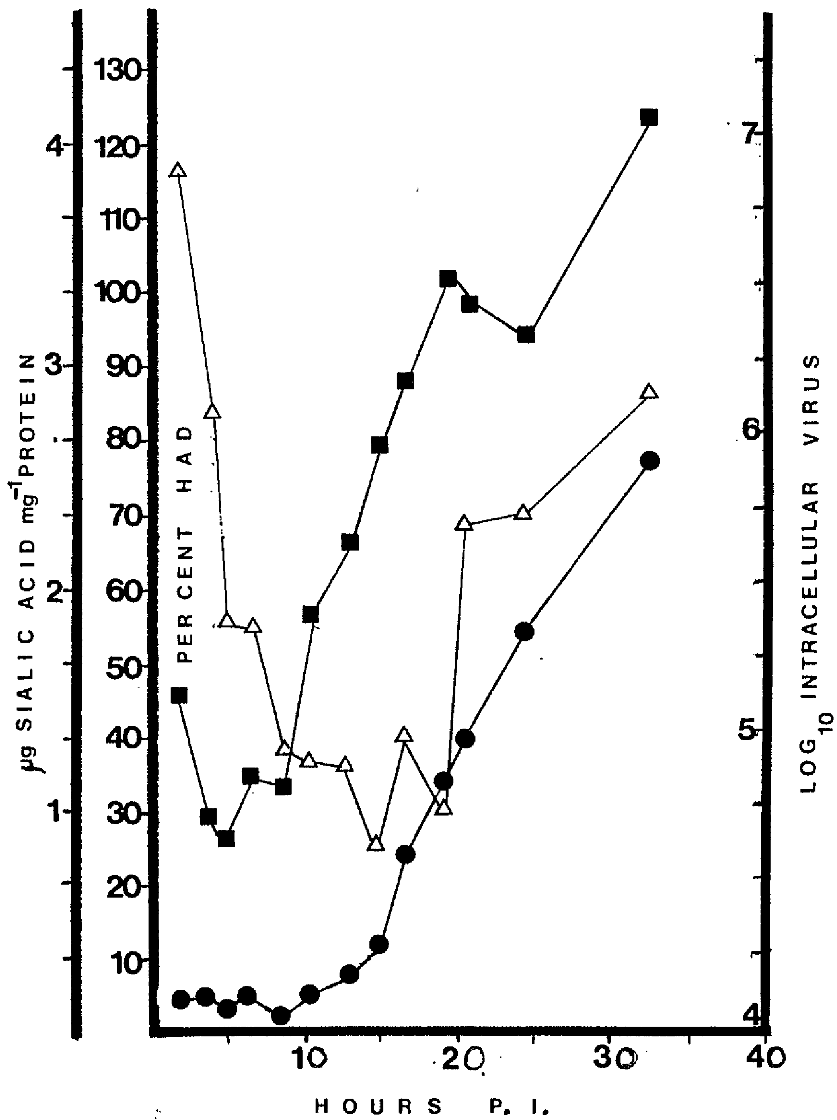
Key:-

■—■ infectious virus ($p.f.u.ml^{-1}$)

●—● haemadsorption (compared to HAD at 48 h p.i.* to vaccinia-infected cells)

△—△ sialic acid ($\mu g mg^{-1}$ protein)

*This experiment was carried out concurrently with that described in Figure 2 (section B(i)), but here, results of the 48 h samples for HAD, infectious virus and sialic acid are not included since no sample was available for sialic acid estimation.



μg SIALIC ACID mg^{-1} PROTEIN
4
3
2
1
PERCENT HAD
130
120
110
100
90
80
70
60
50
40
30
20
10
LOG$_{10}$ INTRACELLULAR VIRUS
7
6
5
4
10
20
30
40
HOURS P. I.

membrane as a dynamic structure (Singer and Nicholson, 1972) has stimulated studies of the "organelle" in a form free from other contaminating cellular membraneous material. The membrane may be harvested as an entire sheet or "ghost" (Warren, Glick and Nass, 1966) or as vesicles produced by pressurised disruption of the cells (Steck and Wallach, 1970). In the former case, difficulties in detaching subcellular organelles and attached submembraneous systems may arise, while in the latter case the resulting "soup" of vesicular material may be partially resolved by use of Ficoll gradients (Wallach and Lin, 1973). In the case of HeLa cells, the isolation of intact ghosts has generally been the method of choice.

Several methods of isolation of plasma membranes were attempted, however the combination of sudden pH change in low molarity buffer with gentle mechanical shear regularly yielded intact ghosts in high proportion, while causing minimal damage to other cell organelles. Other methods attempted included the disruption of cells by osmotic lysis, and mechanical shear in the presence of Zn^{2+} ions which stabilises the membranes by hardening.

Osmotic lysis (Graham, J., personal commun.). Cells were transferred from PBS to a solution of 5 mM Tris buffer pH 7.4 containing 1M glycerol at $0^{o}C$. After 30 min the swollen cells were pelleted and resuspended in a solution containing 0.25M sucrose, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM EDTA (pH 7.4). However, the resultant cell rupture was too severe to preserve the plasma membrane in the intact state.

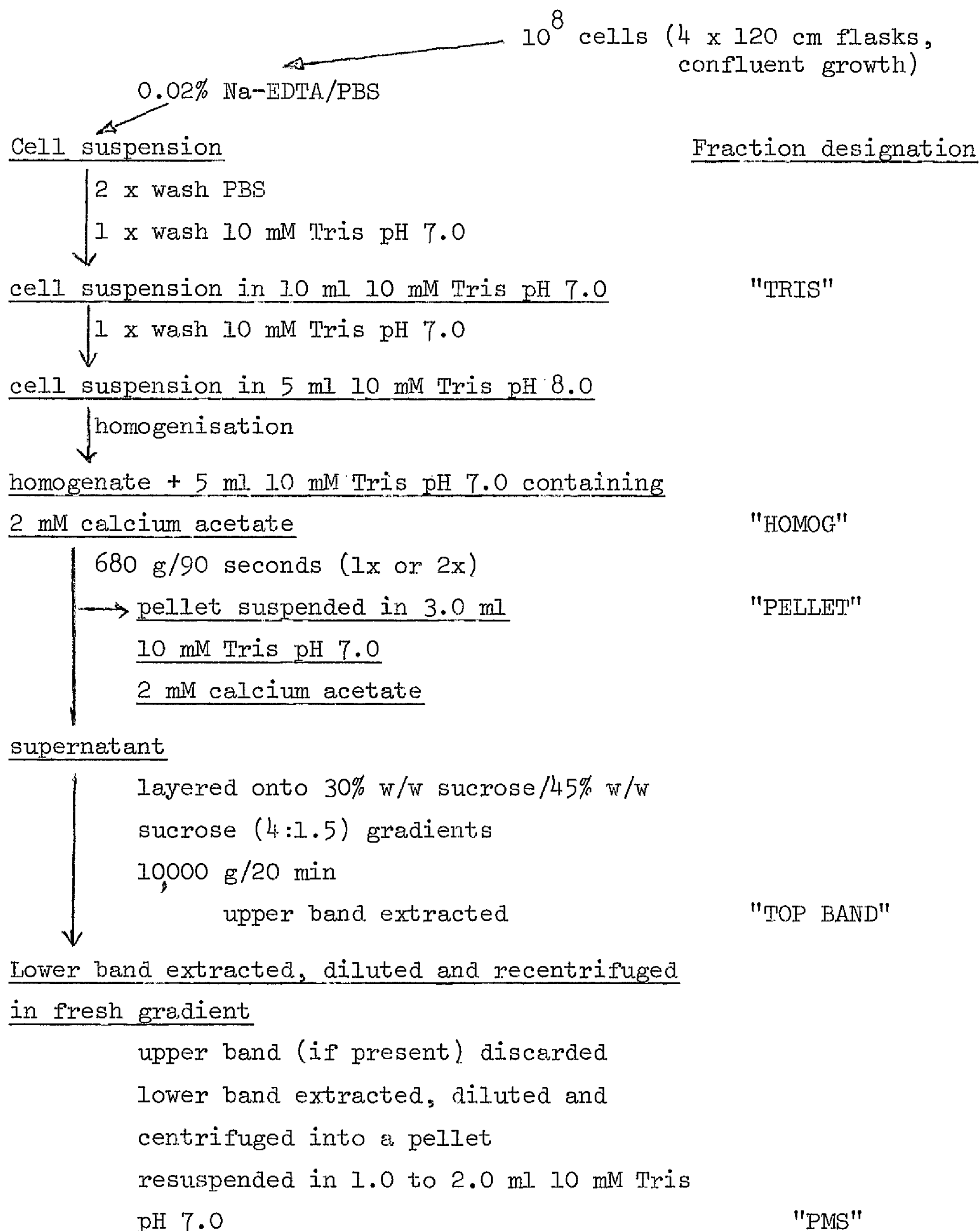
Homogenisation in the presence of Zn^{2+} ions (Warren *et al*, 1966). Cells

washed in PBS were allowed to swell in 10 volumes of 2 mM $ZnCl_2$ for 10 min at room temperature, then for a further 20 min at 0°C in fresh 2 mM $ZnCl_2$ solution. Homogenisation, using a Teflon plunger, was continued until >70 per cent of cells appeared ruptured. However, such breakage only occurred after excessive homogenisation (circa 200 strokes) probably due to the hardening effect of the Zn^{2+} ions on the membranes. The procedure was not carried beyond this point.

An alternative method employing Zn^{2+} ions, that of Brunette and Till (1971) was also attempted with little success. Again hardening of the cell membrane with Zn^{2+} made the cells less amenable to breakage by homogenisation. Brunette and Till modified the original Warren <u>et al</u> method to include separation of the plasma membrane fraction in a two-phase polymer system of polyethylene glycol 6000 and Dextran T500. In this work the band obtained at the phase interface contained no intact ghosts or any identifiable organelles.

The eventual method of isolation provided a plasma membrane fraction identifiable as such by phase contrast microscopy and devoid of any gross contaminating material (Figure 11). Initial transfer of washed cells in 10 mM Tris-HCl pH 7.0 to the same buffer at pH 8.0 resulted in an outward expansion of the cell membrane to three times the normal cell diameter leaving a distinct, centrally situated nucleus. Lengthy incubation in this buffer resulted in gradual cell rupture even at 0°C. Thus the initial weakening of the cell membrane was achieved by the pH change. Complete rupture was achieved by administering only one or two full strokes of the homogeniser. In this respect, the clearance between the plunger and barrel was critical

Figure 11 : Purification of a plasma membrane-rich fraction from monolayer HeLa cells



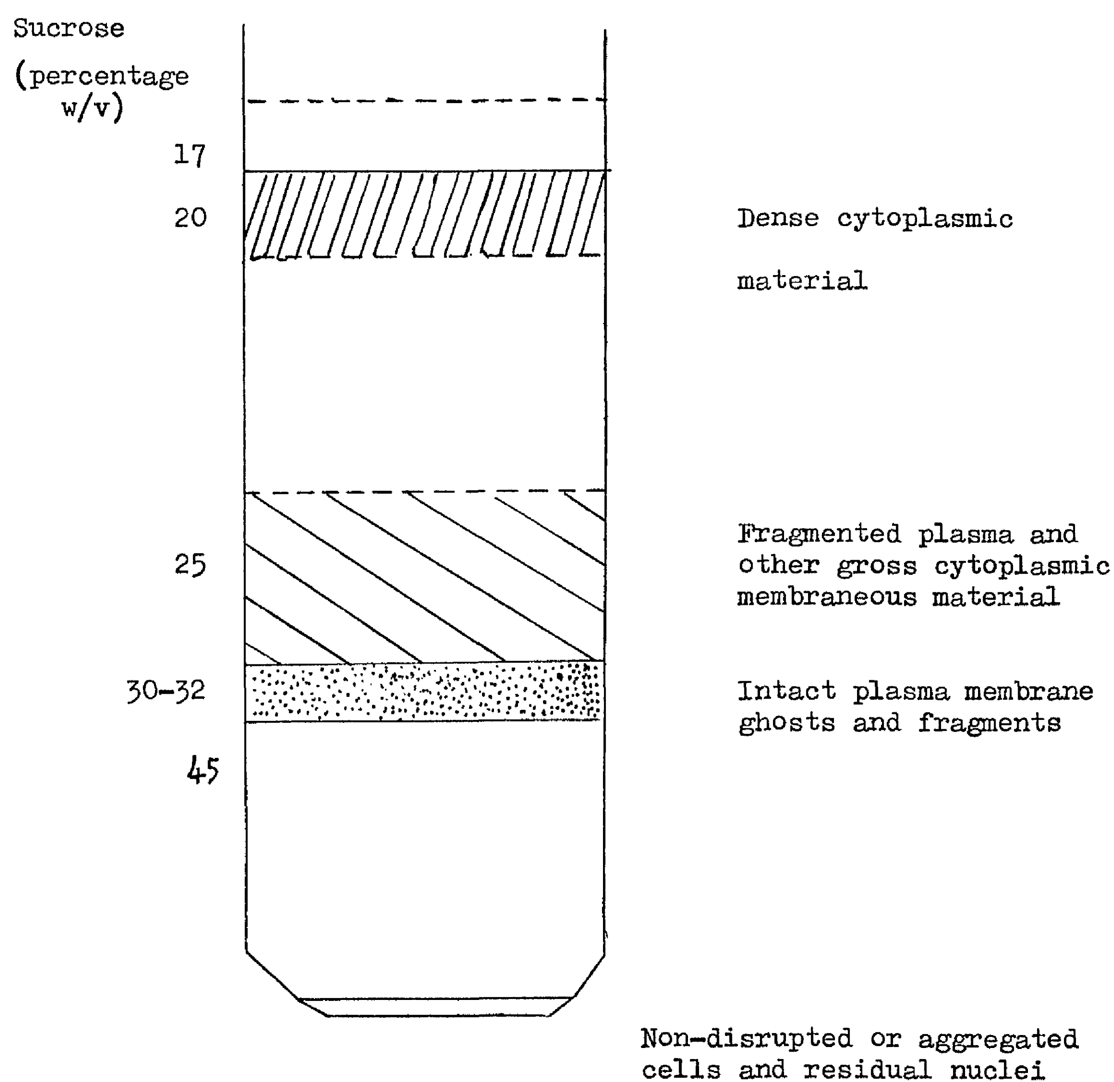
and a reproducible degree of homogenisation could be achieved using a plunger/barrel clearance of 0.05 mm. The liberated nuclei were stabilised by the addition of Ca^{2+} ions which also strengthened the fragile ghosts in the homogenate. Failure to include this step resulted in nuclear lysis and release of DNA.

Centrifugation at 680 g for 90 seconds was sufficient to pellet most nuclei and unbroken cells. Some larger ghosts were also sedimented but the majority remained in the supernatant. Resuspension of the pellet to recover these ghosts tended to enhance nuclear breakage; the increased contamination by nuclear material of any ghosts further separated from the pellet was considered to be too great to justify re-processing.

High speed centrifugation (10,000 g) of the supernatant on sucrose density gradients resulted in the banding pattern illustrated in Figure 12. Bands were recovered either by piercing the gradient tube and collecting fractions or by extracting via the top of the gradient using a syringe. The upper zone consisted of cytoplasmic sap which failed to pass into the sucrose gradient. At the sucrose barrier, a sharper band was found, consisting of cellular organelles, possibly lysosomes and cytoplasmic membrane fractions. In a third diffuse area, towards the 30%/45% w/v sucrose barrier, was found fragmented membraneous material. This area merged with a distinct band found at the interface. Here, with additional fragmented membraneous material, intact sheets of plasma membrane were found, as described by Atkinson (1973). Occasionally some material passed through the gradient forming a pellet at the bottom of the tube.

Figure 12 The distribution of cellular material on a sucrose density gradient during purification of HeLa cell plasma membranes

Fractions obtained from sucrose density gradients were examined by phase contrast microscopy.



This mainly comprised nuclei and whole cells, present as contaminating material in the 680 g supernatant. The fragmented material found in the plasma membrane band could be removed by repeating the density gradient centrifugation.

Aliquots of fractions retained during the purification procedure were assayed for the presence of various marker enzymes.

(ii) Characterisation by assay of marker enzymes

(a) Assay of Na^+, K^+-ATPase. The potassium-dependent, ouabain-inhibited phosphatase is believed to be a composite part of the sodium-potassium-dependent adenosine triphosphatase (Na^+, K^+-ATPase) (Dahl and Hokin, 1974). This, in turn, has been reported to be active in the plasma membrane of all cells, functioning initially as a "pump" in the active transport of metabolites, as well as Na^+ and K^+ ions. The localisation in, and confinement of, the activity to the plasma membrane provides an excellent specific marker for determining the purity of plasma membrane fractions (Wallach and Lin, 1973). Another activity, 5'-nucleotidase, has been regarded as a marker enzyme for HeLa cell plasma membranes (Bosmann, Hagopian and Eylar, 1968), however its specificity has been questioned (Johnsen et al, 1974). Na^+, K^+-ATPase activity has been detected in HeLa cell plasma membrane ghosts or fragments (Bosmann et al, 1968; Boone et al, 1969).

However, despite attempting several assays designed to detect ATPase by measuring activity in the presence and absence of Na^+ and K^+ ions, or by inhibition with ouabain, no detectable levels of Na^+, K^+-ATPase were found. Among the methods attempted were those

of Quigley and Gotterer (1969), Wallach and Kamat (1966), Hemminki (1973) and Izutsu, Siegel and Brisson (1974). Failure to observe any activity by these methods was attributable possibly to very low inherent levels, to inactivation or inhibition by the original treatment of the monolayer cells with EDTA or to a combination of these factors.

(b) Assay of K^+-dependent phosphatase. The K^+-dependent phosphatase activity was mainly associated with the fraction identified morphologically as that containing purified plasma membranes (Table 21). The low levels in the nuclei-rich (PELLET) fraction and 680 g supernatant fraction were taken as an indication of the amount of non-plasma membrane protein present. With respect to the supernatant fraction the majority of the protein was of cytoplasmic origin and only a small fraction can be regarded as plasma membrane protein.

(c) Assay of phosphodiesterase. Phosphodiesterase activity has been widely used as a specific marker enzyme for purified plasma membrane fractions (Bosmann et al, 1968; Rufeger, Tellhelm and Kroker, 1974; Gavard, de Lamirande and Karasaki, 1974; Durham, Galanti and Revis, 1975).

In this work activity was detectable only in the presumed plasma membrane fraction and even then only at low levels (Table 22).

(d) Assay of NADH-diaphorase. NADH-diaphorase or oxidase is located specifically in the membranes of the endoplasmic reticulum (Wallach and Kamat, 1966) and catalyses the oxidation of reduced NAD (NADH) by various acceptors. The activity has normally been assayed

Table 21 Distribution of potassium-dependent p-nitrophenyl phosphatase activity in cell fractions during purification of HeLa cell plasma membranes

Enzyme activity, monitored by spectrophotometric measurement of the release of p-nitrophenol from the substrate, p-nitrophenyl phosphate (tris), is expressed as the difference in number of nmoles of substrate hydrolysed in the presence or absence of K^+ ions in the reaction mixture, per μg of cellular protein per 30 minutes of incubation time. The conversion from $\Delta E_{410\ nm}^{1\ cm} \pm K^+$ to nmoles hydrolysed is shown in Figure 13.

Fraction	Average Δ nmoles pNpp hydrolysed per μg protein per 30 min when K^+ ions were present in the reaction mixture
TRIS	0.095
HOMOG	0.072
PELLET	0.063
SUPT	0.058
TOP BAND	0.102
PMS	0.286

Figure 13 Standard curve for determining hydrolysis of p-nitrophenylphosphate

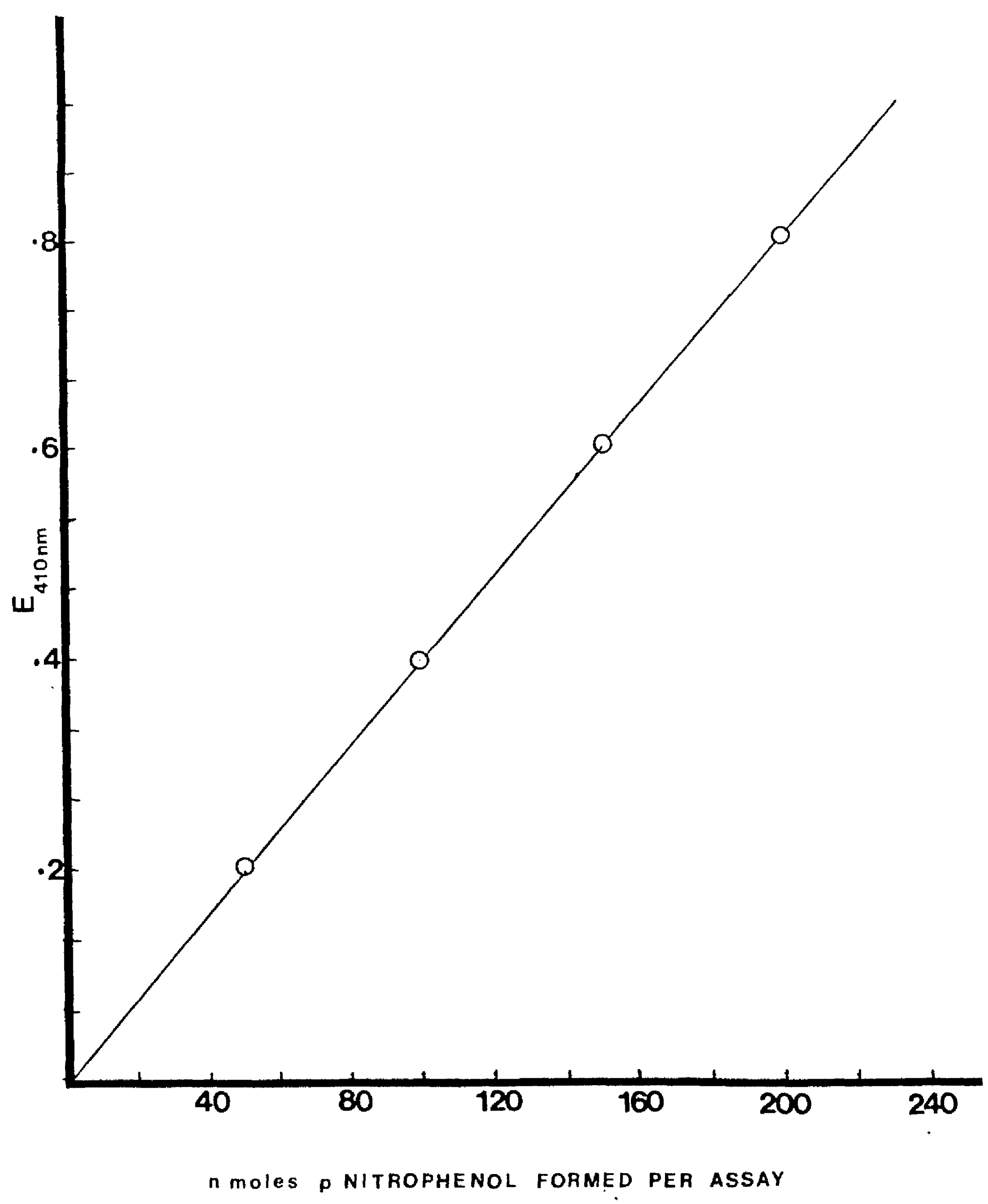

·8
·6
E_{410nm}
·4
·2
40
80
120
160
200
240
n moles p NITROPHENOL FORMED PER ASSAY

Table 22 Distribution of phosphodiesterase activity in cell fractions during purification of HeLa cell plasma membranes

The release of p-nitrophenol from calcium bis-p-nitrophenyl phosphate was used as a measure of enzyme activity, by monitoring the $\Delta E^{1\ cm}_{410\ nm}$ of the reaction mixture. $\Delta E^{1\ cm}_{410\ nm}$ units was converted to μmoles p-nitrophenol formed per mg protein per 30 min incubation by means of a standard curve (Figure 13).

Fraction	Average μmoles p-nitrophenol formed per mg protein per 30 min
TRIS	Undetectable (< 0.01)
HOMOG	Undetectable
PELLET	Undetectable
SUPT	Undetectable
TOP BAND	Undetectable
PMS	2.28

when that of glucose-6-phosphatase, also a specific endoplasmic reticulum marker, was too low to measure. NADH-diaphorase is therefore a negative marker with respect to plasma membranes, and a high purity of plasma membrane preparation is suggested by the absence of any appreciable NADH-diaphorase activity.

Figure 14 shows the curves of oxidation of NADH by each cell fraction during incubation with the reaction mixture. Potassium ferricyanide acted as the electron acceptor. The reaction rates were comparable in most fractions, however the fraction from the top of the sucrose density gradient, containing presumably the bulk of the intracellular membranes, had the fastest reaction rate. There was a complete absence of activity in the purified plasma membrane fraction.

(e) Assay of β-glucuronidase. The enzyme β-glucuronidase, present predominantly in the lysosomal fraction of cells (Allison and Sandelin, 1963), has been used as a 'negative' marker during the preparation of plasma membrane fractions (Marique and Hildebrand, 1973; Lin *et al*, 1976). The enzyme catalyses the reaction, β-D-glucuronide + water → an alcohol + D-glucuronate. Enzyme activity was measured spectrophotometrically by the release of free phenolphthalein from phenolphthalein-β-glucuronide (Allison and Sandelin, 1963).

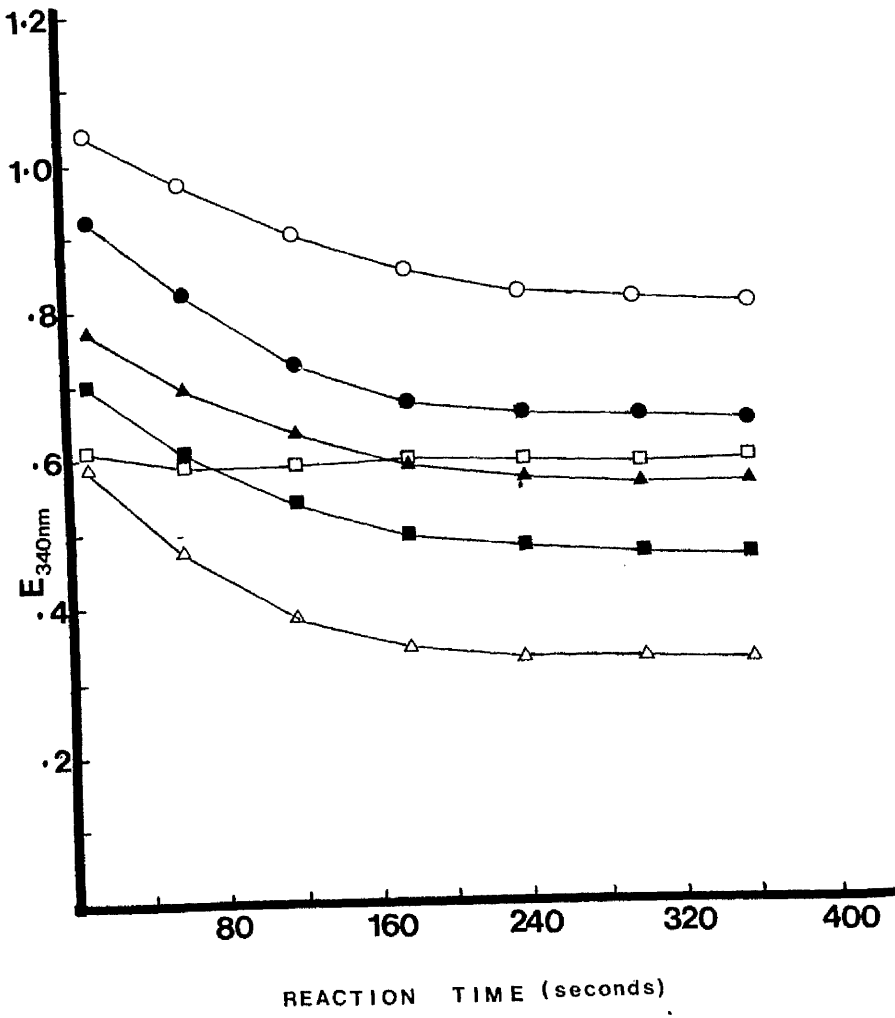
Most activity was again found in the "top" fraction (Table 23), where the majority of the lysosomal material and other subcellular material would be expected to have concentrated. Moderate amounts

Figure 14 The rate of oxidation of NADH by cell fractions obtained during the purification of a plasma membrane fraction from HeLa cells

Oxidation was monitored by following the gradual conversion of NADH to NAD by the cellular enzyme, NADH diaphorase, measureable spectrophotometrically by a decrease in $E^{1\ cm}_{340\ nm}$.

Key:-

	cell fraction
○—○	"PELLET"
●—●	"TRIS"
▲—▲	"HOMOG"
■—■	"SUPT."
□—□	"PMS"
△—△	"TOP BAND"



1·2
1·0
·8
·6
·4
·2
E_{340nm}
80
160
240
320
400
REACTION TIME (seconds)

Table 23 Distribution of β-glucuronidase activity in cell fractions during purification of HeLa cell plasma membranes

Enzyme activity was monitored by spectrophotometric measurement of the release of free phenolphthalein from phenolphthalein-β-glucuronide. The results are expressed as the $\Delta\ E^{1\ cm}_{545\ nm}$ per mg protein during an incubation period of 30 min.

Fraction	$\Delta E^{1\ cm}_{545\ nm}$ units per mg protein per 30 min
TRIS	0.22
HOMOG	0.30
PELLET	0.08
SUPT	0.25
TOP BAND	0.38
PMS	Undetectable ($<$ 0.04)

of activity were found in other fractions but none was detected in the plasma membrane fraction.

(iii) Characterisation by specific uptake of ^{3}H-L-fucose

In HeLa cells it has been reported that fucose is required as a constituent of cell glycoproteins (Trujillo and Gan, 1971; Atkinson and Summers, 1971; Atkinson, 1973). Much of the fucose can be accounted for in the plasma membrane (Shen and Ginsburg, 1968; Bosmann, Hagopian and Eylar, 1969; Atkinson and Summers, 1971; Nowakowski, Atkinson and Summers, 1972) and this may also be true in other cell systems (Bennett and Leblond, 1970; Gahmberg, 1971). The relative specific activities of cell fractions may therefore indicate the purity of plasma membrane preparations (Atkinson, 1973).

Exponentially growing HeLa cells (1×10^{7}) were incubated for 24 h with SFM containing 80 μCi ^{3}H-L-fucose, and plasma membrane fractions prepared. After removing the cells from the glass and washing twice, 4.7 per cent of the original radioactivity added to the maintenance medium was associated with the cells. Earlier experiments indicated that approximately 25 per cent of the original ^{3}H counts applied could be eluted from cells during treatment with EDTA (to strip the cells from the monolayer state) and during subsequent washes. Trypsin treatment is known to release up to 31 per cent of total cell fucose from monolayers of intact HeLa cells (Shen and Ginsburg, 1968) and low frequency ultrasonication non-specifically released up to 26 per cent of total cell fucose during detachment from the monolayer state (Payne *et al*, 1973).

HeLa cell plasma membranes contained in the supernatant from a 680 *g* centrifugation of the cell homogenate were purified by centrifugation on a 30 per cent/45 per cent w/v sucrose gradient. The distribution of

a) total ^{3}H-L-fucose label and

b) TCA-precipitable ^{3}H-L-fucose label

in the sucrose gradient was compared with the microscopical distribution of purified plasma membranes (Table 24). The TCA-precipitable counts measured the protein-bound label while the total counts in addition accounted for any free L-fucose not incorporated into glycoprotein.

By phase contrast microscopy, intact plasma membrane ghosts were consistently found at the 30 per cent/45 per cent w/v sucrose interface. The major peaks of both TCA-precipitable and total counts were coincident at this point in the gradient (Figure 15). From Table 24, the TCA-precipitable counts per μg protein in the peak fraction (Fraction 8) represented a 3.5-fold purification from the original cell suspension ("Tris") and 7.56 per cent of TCA-precipitable ^{3}H-L-fucose label present in the cell suspension prior to homogenisation was recovered. This and the protein recovery of 3 per cent compared favourably with the results of Atkinson (1973) who reported a total protein recovery in terms of tritium label of 4 to 7 per cent in his purified plasma membrane fraction. Thus by the criteria employed, phase contrast microscopy, enzyme assay and ^{3}H-L-fucose uptake, the cell fraction banding at the 30 per cent/45 per cent interface in a sucrose density gradient, was considered an acceptably purified preparation of HeLa cell plasma membranes in the form of intact ghosts.

Table 24 Distribution of ^{3}H-L-fucose label in cell fractions during purification of HeLa cell plasma membranes

Fraction	Counts min^{-1} μg $protein^{-1}$	
	Total	TCA Precipitable
TRIS	150	70
HOMOG	128	67
SUPT	138	72
PELLET	90	57
TOP BAND (Gradient Fraction 3)	116	64
PMS (Gradient Fraction 8)	288	249

The complete distribution of ^{3}H-L-fucose label after centrifugation of the 680 g supernatant on a sucrose density gradient is shown in Figure 15.

Figure 15 The distribution of ^{3}H-L-fucose in sucrose density gradients during a stage of purification of HeLa cell plasma membranes

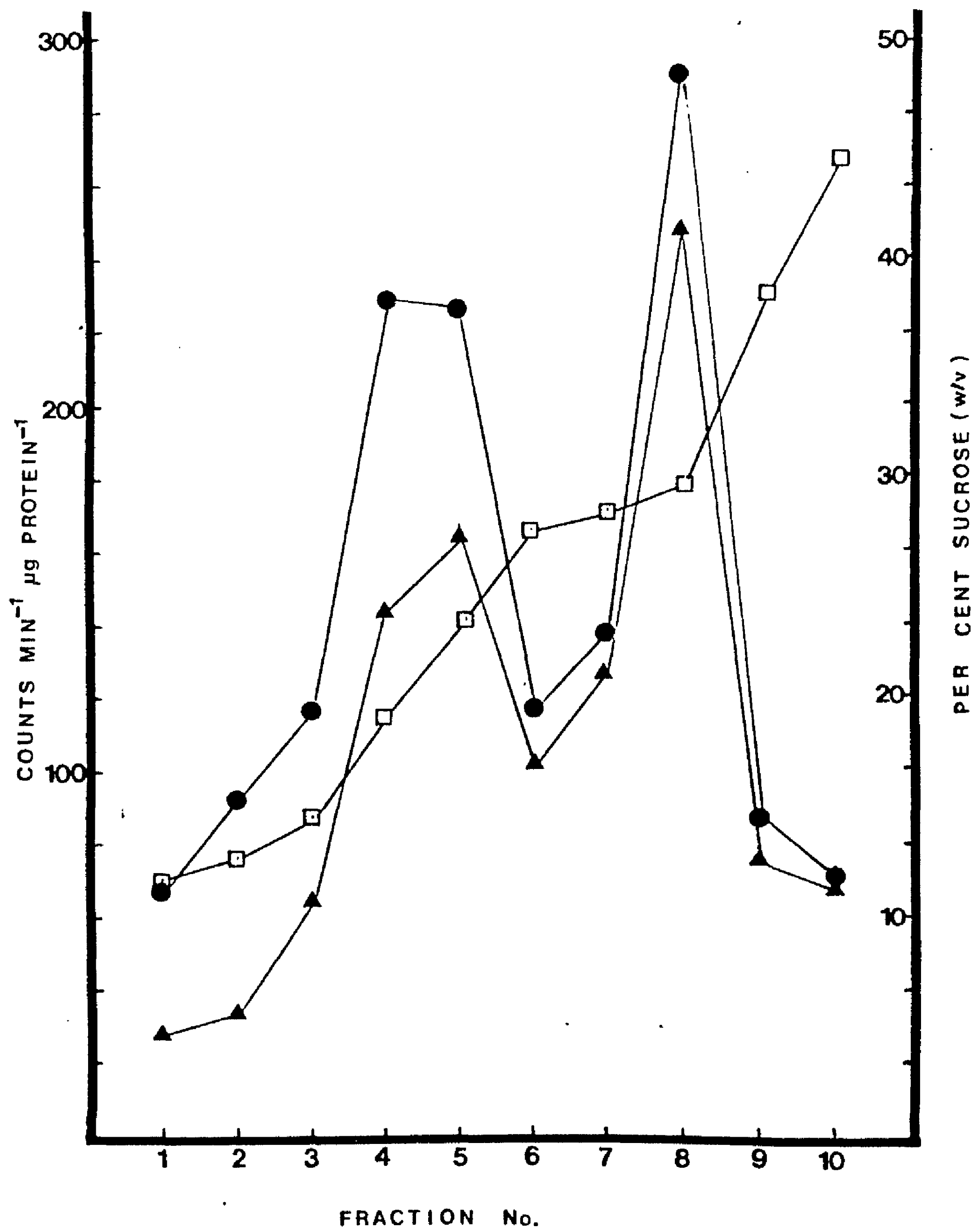
Cells were prelabelled in the monolayer state with ^{3}H-L-fucose before fractionation as described in the text. Density gradient centrifugation of the 680 g supernatant in sucrose was followed by fractionation of the gradient and estimation of the radioactive label distribution. The total counts, i.e. those incorporated into proteins plus "free" ^{3}H-L-fucose were estimated directly on the fractions. An estimation was also made of the protein-bound label, as determined by precipitation with trichloroacetic acid (TCA) onto filter paper.

Key:-

●—● total counts

▲—▲ TCA precipitable counts

□—□ [sucrose] (per cent w/v).


300
200
100
COUNTS MIN^{-1} µg PROTEIN^{-1}
50
40
30
20
10
PER CENT SUCROSE (w/v)
1
2
3
4
5
6
7
8
9
10
FRACTION No.

(iv) Polyacrylamide gel electrophoresis of cell fractions obtained in the purification procedure

When the fractions obtained by purification of HeLa cell plasma membranes were compared by SDS-PAGE considerable differences were observed (Plate 5). From the complex banding pattern of the cell homogenate (> 50 polypeptides) at least 14 polypeptides were identified in the PM fraction, two of which (molecular weights 39,000 and 45,000 daltons respectively) were major constituents. Five low molecular weight and two high molecular weight polypeptides present in the crude homogenate were concentrated in the 680 g pellet. A number of polypeptides present in the 680 g supernatant but not in the PM fraction were presumably of cytoplasmic origin. One major band of 45,000 daltons was common to all the cell fractions tested.

F. COMPARISON OF PLASMA MEMBRANES FROM UNINFECTED AND VACCINIA-INFECTED HELA CELLS

(i) Polyacrylamide gel electrophoresis

The polypeptide banding patterns of plasma membranes of vaccinia-infected HeLa cells were outwardly very similar to those of plasma membrane fractions of uninfected cells or cells infected in the presence of ara-C except that three additional polypeptides (34,000, 56,000 and 88,000 daltons) were detected (Plate 6).

(ii) Investigation of the possible association of VHA with purified plasma membranes from uninfected HeLa cells

It was postulated by Blackman and Bubel (1972) that the

Plate 5 Sodium dodecyl sulphate polyacrylamide gel electrophoresis of subcellular fractions isolated during purification of plasma membranes from HeLa cells

Samples taken at each stage of plasma membrane purification were analysed by SDS-PAGE. The fractions correspond to those described in Figure 11.

Key:-

1. Cell homogenate
2. 680 g precipitate
3. 680 g supernate
4. Plasma membrane fraction
5. Blank
6. Molecular weight standards

The molecular weight standards used were lysozyme (14,300), ovalbumin (45,000) and human serum albumin (69,000) and the molecular weight scale was calculated from a calibration graph of log molecular weight against migration distance.

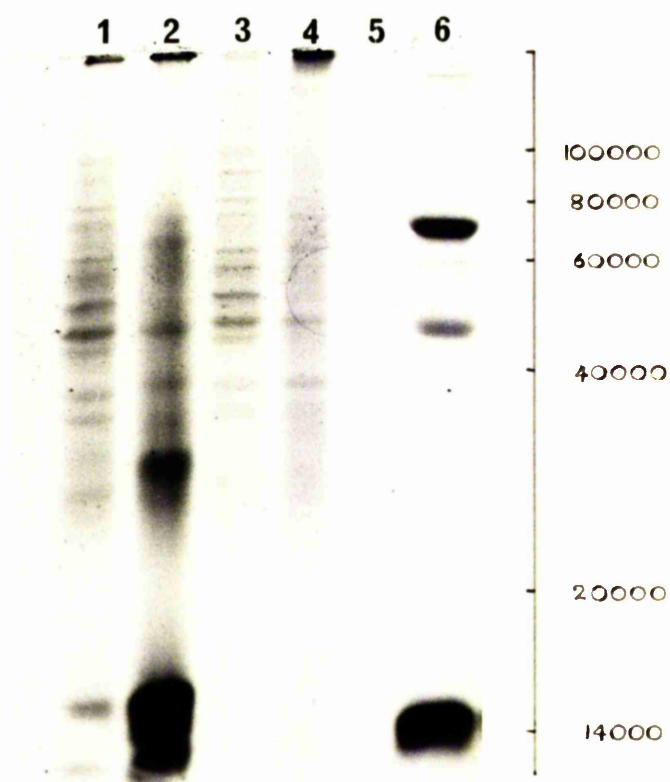
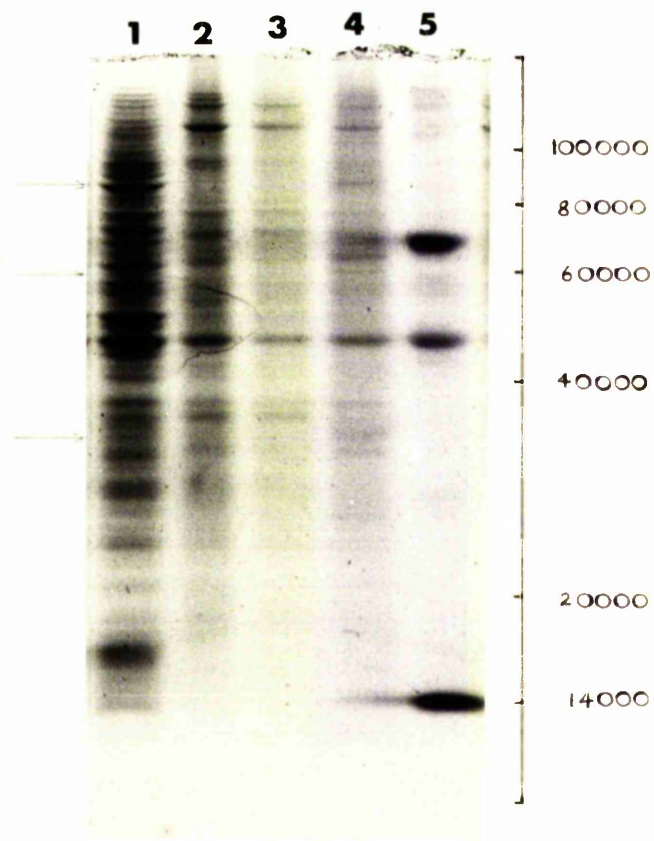

1
2
3
4
5
6
100000
80000
60000
40000
20000
14000

Plate 6 Sodium dodecyl sulphate polyacrylamide gel electrophoretic comparison of purified plasma membranes from uninfected and vaccinia-infected HeLa cells

SDS-PAGE was carried out on a purified plasma membrane fraction from uninfected HeLa cells and on similar fractions from vaccinia-infected HeLa cells (in the presence or absence of ara-C) harvested at 24 h p.i.

Key:-

1. HeLa cell homogenate
2. Plasma membrane fraction:- 24 h vaccinia-infected cells (with ara-C)
3. Plasma membrane fraction:- uninfected HeLa cells
4. Plasma membrane fraction:- 24 h vaccinia-infected cells
5. Molecular weight standards (see Plate 5).


1
2
3
4
5
100000
80000
60000
40000
20000
14000

origin of VHA was the plasma membrane of the virus-infected cell. This was based largely on the finding that isolated plasma membrane ghosts from vaccinia-infected cells could haemadsorb susceptible fowl erythrocytes. It was of interest therefore to establish whether extracts of infected HeLa cells contained free VHA protein which might under suitable conditions associate with plasma membrane ghosts from uninfected cells resulting in formation of functional VHA.

Equal volumes of a preparation of purified plasma membranes (PM), crude VHA and Tris buffer were mixed as below:

a) PM + VHA

b) PM + Tris

c) VHA + Tris.

Each mixture was incubated for 30 min at 37°C, before layering on to a two step gradient (30 per cent/45 per cent w/v, in 10 mM Tris buffer, pH 7.0). The distribution of HA and protein in the three gradients is shown in Figure 16. Interaction between an unbound VHA protein component and purified plasma membranes could lead to a peak of HA activity at the 30 per cent/45 per cent sucrose interface, the location of the plasma membrane band or to a depletion of the PM band. No shift in the HA peak or loss of HA activity was effected by isolated HeLa cell plasma membranes under these conditions and the HA peaks were coincident. Plasma membrane ghosts were found by microscopy in fractions 3 and 4.

(iii) <u>Experiments with antisera to plasma membranes of uninfected and vaccinia-infected HeLa cells</u>

Figure 16 Investigation of the possible association of VHA with purified plasma membranes from uninfected HeLa cells

Plasma membranes (from 8×10^7 cells) purified on sucrose density gradients as previously described, VHA (a crude extract of vaccinia-infected cells, HA titre 1/256) and 10 mM Tris buffer, pH 7.0 were mixed together in dual combinations to determine whether the buoyant density of VHA or of plasma membranes in a 30 per cent/45 per cent w/v sucrose gradient might be affected by possible interaction. After centrifugation (10,000 g for 20 min) 40 drop fractions were collected after piercing the bottom of the tube and assayed for HA, protein and sucrose concentration.

Key:-

○—○ PM + VHA ($1/HA_{50}$ titre)

●—● PM + VHA (protein, $\mu g\ ml^{-1}$)

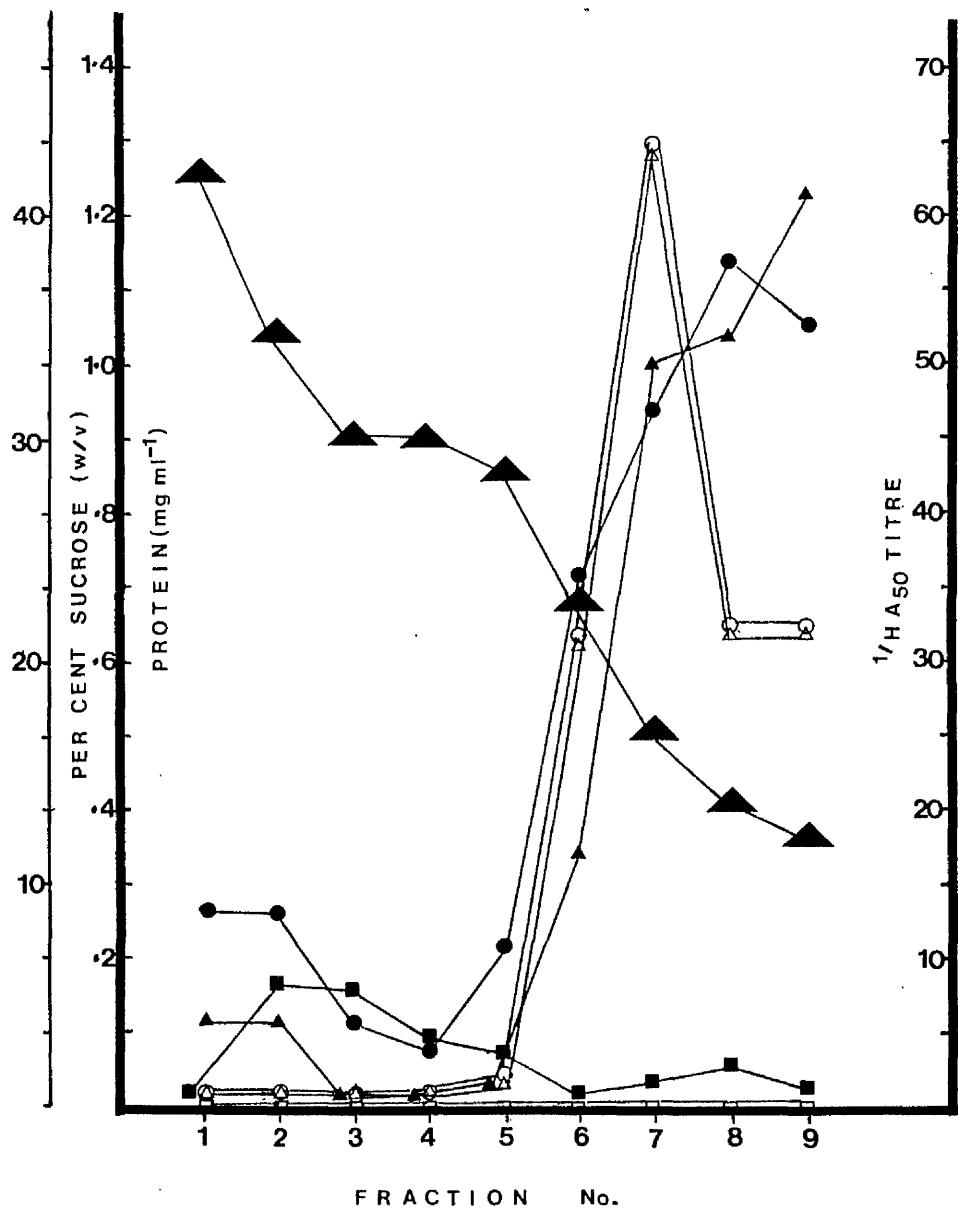
□—□ PM + Tris ($1/HA_{50}$ titre)

■—■ PM + Tris (protein, $\mu g\ ml^{-1}$)

△—△ VHA + Tris ($1/HA_{50}$ titre)

▲—▲ VHA + Tris (protein, $\mu g\ ml^{-1}$)

▲—▲ sucrose (per cent w/v) (average).



PER CENT SUCROSE (w/v)
PROTEIN (mg ml^{-1})
$^{1}/_{H}$ A$_{50}$ TITRE
FRACTION No.
40
30
20
10
1·4
1·2
1·0
·8
·6
·4
·2
70
60
50
40
30
20
10
1
2
3
4
5
6
7
8
9

(a) Immunofluorescence: Fluorescence studies performed with antiVACγG revealed the presence of specific VICSA (page 73). Rabbit antiserum to HeLa cell plasma membranes from uninfected or vaccinia-infected cells were tested in an attempt to demonstrate specific cell surface fluorescence. The experimental conditions were as previously outlined using heat inactivated antisera or γG fractions.

Antisera to plasma membranes from uninfected cells (AUPM) reacted with uninfected cells giving distinct membrane fluorescence (Plates 7a,b) and antiserum to plasma membranes from vaccinia-infected cells (AIPM) reacted to show weak cell membrane fluorescence. At 2 h p.i., AIPM showed strong membrane fluorescence but only with cells displaying virus-induced cell rounding (Plates 7c,d). Uninfected cells in the same field showed little or no reaction. The strong membrane fluorescence on vaccinia-infected cells was also observed 24 h p.i. (Plates 7e,f) or when ara-C was present in the medium (Plates 7g,h). Thus the antiserum to plasma membranes from 24 h vaccinia-infected cells, detected virus-induced cell surface changes as early as 2 h p.i., in the absence of viral DNA synthesis.

Since AIPM sera 34 and 35 were raised to plasma membranes of vaccinia-infected HeLa cells, antibodies to host components would be expected to be present. To circumvent the reaction with host components AIPM sera 34 and 35 and antiVACγG were tested against vaccinia-infected RK 13 cells. Following addition of FITC-goat anti-rabbit γ-globulin membrane fluorescence similar to that found with infected HeLa cells was evident at 2 h p.i. and 24 h p.i. with all sera. (Results not shown).

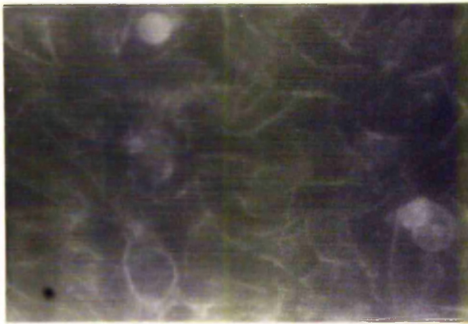
Plate 7 Immunofluorescence studies using antisera to plasma membrane fractions from uninfected or vaccinia-infected HeLa cells

Monolayers of unfixed HeLa cells, uninfected or infected with vaccinia, were incubated with either AUPM (# 27) or AIPM (#35) serum at various times after infection. After washing in PBS the monolayers were treated with FITC-labelled sheep anti-rabbit globulin and again washed. The presence and location of specific fluorescence was determined by comparing fields photographed at X400 magnification under phase contrast and UV light.

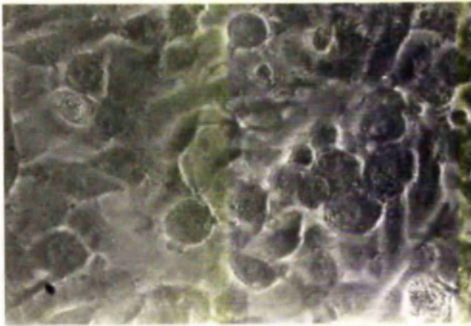
Plate	Cells	Antiserum; illumination
Plate 7a:	uninfected cells with AUPM (# 27);	phase contrast
" 7b:	" " " " "	UV
" 7c:	vaccinia-infected cells (2 h p.i.)	with AIPM (#35); phase contrast
" 7d:	" " " "	with AIPM (#35); UV
" 7e:	" " " (24 h p.i.)	with AIPM (# 35); phase contrast
" 7f:	" " " "	with AIPM (# 35); UV
" 7g:	" " " (24 h p.i. in the presence of ara-C);	with AIPM (# 35); phase contrast
" 7h:	" " " " "	UV

Plate magnification 1.8X.

a



b



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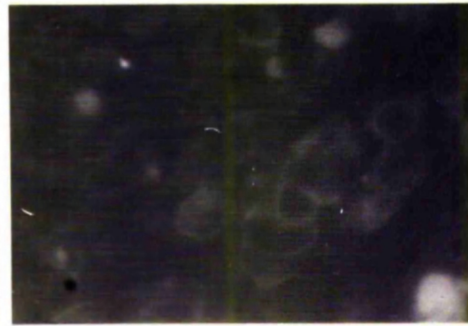

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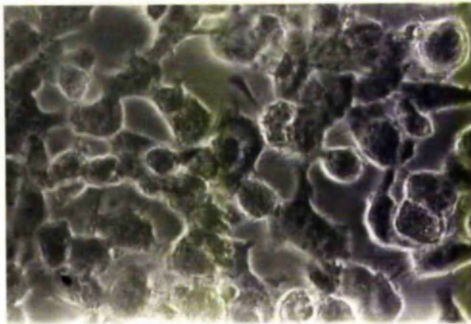

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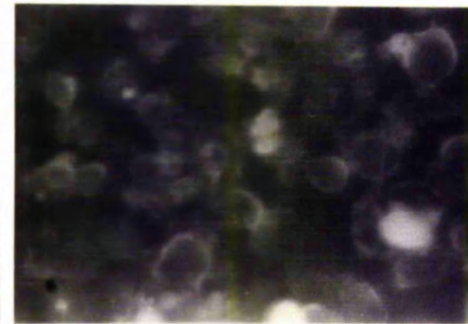

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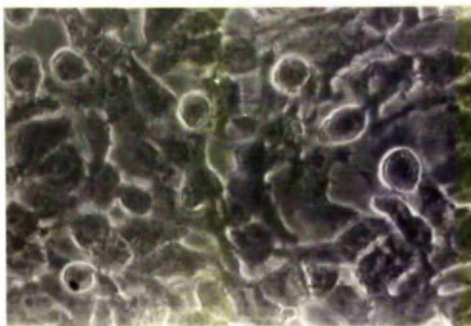

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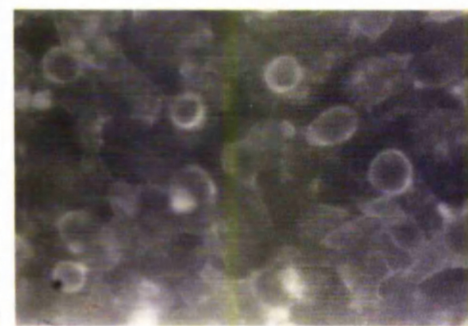

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(b) Immune haemadsorption. Sera (AUPM and AIPM) were tested with both virus-infected and uninfected HeLa cells (Table 25); AUPM serum bound similarly to uninfected and vaccinia-infected cells at 2 h p.i. although preferential binding of AIPM serum to virus-infected monolayers could be demonstrated at this time. By 24 h, after correction for the non-specific red cell binding increase, the AIPM serum still showed preferential and increased binding to virus-infected monolayers.

(c) Blocking of VHA-mediated haemadsorption. Undiluted antisera were tested for their ability to block HAD. The results (Table 26) showed that all antisera tested inhibited HAD. Anti-HeLa serum reduced HAD to 50 per cent and still displayed some inhibition when diluted to 1/100; AIPM (rabbit 35) almost completely inhibited binding of turkey erythrocytes.

(d) Other properties of the antisera. The results of HAI, complement fixation and neutralisation titrations of the various antisera are shown in Table 27.

As expected, in all cases antiVACγG was the most potent antiserum tested, but this was magnified as the preparation was a concentrated γ-globulin fraction. Antisera to HeLa plasma membranes and to whole cells had no HAI activity beyond a nonspecific inhibition of red cell pelleting.

Antisera 34 and 35 showed a considerable difference in ICV neutralising titres but similar ECV neutralising titres. On immuno-diffusion, the two AIPM sera and antiVACγG showed identity reactions

Table 25 Detection of vaccinia-specific antigens on the surface of infected HeLa cells by immune haemadsorption using antiserum to plasma membranes from vaccinia-infected HeLa cells

Monolayers were assayed as described in Materials and Methods (p 59) as an integral part of that experiment.

Antiserum (rabbit)	Average $E_{410\ nm}^{1\ cm}$ of lysate (corrected)			
	Uninfected cells		Vaccinia-infected cells	
	2 h p.i.	24 h p.i.	2 h p.i.	24 h p.i.
NγG	0.172	0.184	0.161	0.182
anti-HeLa	1.050	1.070	n.t.	n.t.
antiVACγG	0.165	0.184	0.285	0.600
(AUPM) (rabbit No. 31)	0.232	0.226	0.241	0.209
(AIPM) (rabbit No. 35)	0.261	0.237	0.310	0.774

Table 26 The inhibition of haemadsorption to vaccinia-infected cells by antisera

Rabbit antiserum	Haemadsorption (%)*	
	Infected cells	Uninfected cells
AUPM (rabbit No. 31)	51, 56, 60	3, 3, 2
AUPM (rabbit No. 27)	69, 80, 65	2, 2, 1
AIPM (rabbit No. 35)	7, 6, 4	1, 0, 1
AHELA (rabbit No. 29)	51, 47, 45	1, 2, 4
NORMAL RABBIT γG	100, 100, 100	2, 0, 1

* Compared to monolayers treated with NγG.

against vaccinia soluble antigen preparations grown in HeLa cells (HVSA) and rabbit dermis (RVSA) although the AIPM sera showed fewer lines against the latter (Plates 8a,b). Antisera 27 and 31 (AUPM) also reacted with HVSA (Plate 8c) and with a soluble antigen preparation from uninfected HeLa cells (HeSA) (Plate 8d). Sera 34 and 35 showed no reaction with HeSA (Plate 8d) and no identity reactions with sera 29 and 31 (Plates 8c,d). This may have resulted from a form of antigenic competition between HeLa and vaccinia-specific antigens where the immunised rabbits may have reacted preferentially only to one immunogen (Taussig, 1973).

On plate 8b, the reaction between serum 26 and serum 35 was presumably due to allotypic differences between the animals.

In attempts to identify the VHA-specific precipitin line HVSA was absorbed at 37°C for 1 hour with excess turkey red cells or with human cells. When this antigen preparation, with no residual HA activity, was compared with control antigen (HA_{50} titre 1/256) using antiVACγG and AIPM 35 serum, no loss of precipitinogens was detected (Plates 8e,f).

Table 27 Complement fixing, neutralising and haemagglutination-inhibition titres of antisera

Antiserum	CF_{50} titre[1]	ND_{50} titre[2]		HAI_{50} titre
		ECV	ICV	
NγG	< 1/2	< 1/2	< 1/2	< 1/2
antiVACγG	1/64 - 1/128	1/16,000	1/22,000	1/16,000
AIPM (No. 34)	n.t.	1/700	1/700	1/128
AIPM (No. 35)	1/16	1/300	1/10,000	1/512
AUPM (No. 27)	n.t.	n.t.	n.t.	1/4
AUPM (No. 31)	n.t.	n.t.	n.t.	1/4
antiHeLa (No. 26)	n.t.	n.t.	n.t.	1/2

n.t. = not tested

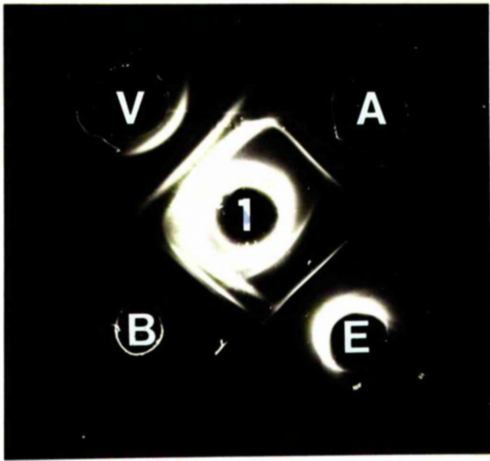
[1]complement fixation titre

[2]neutralising antibody titre.

Plate 8 Immunodiffusion studies with antisera to plasma membranes from uninfected and vaccinia-infected HeLa cells

Key:-

		≡	
Antisera	A	≡	AIPM (No. 34)
	B	≡	AIPM (No. 35)
	C	≡	AUPM (No. 27)
	D	≡	AUPM (No. 31)
	V	≡	antiVACγG
	E	≡	anti-HeLa (No. 26)
	F	≡	anti-HeLa (No. 29)
Antigens	1	≡	vaccinia/HeLa cell extracted soluble antigen (HVSA)
	2	≡	soluble antigen extract of vaccinia-infected rabbit dermis (RVSA)
	3	≡	soluble antigenic extract of HeLa cells. (HeSa)
	4	≡	HVSA pre-absorbed with human "O" erythrocytes
	5	≡	HVSA pre-absorbed with turkey erythrocytes



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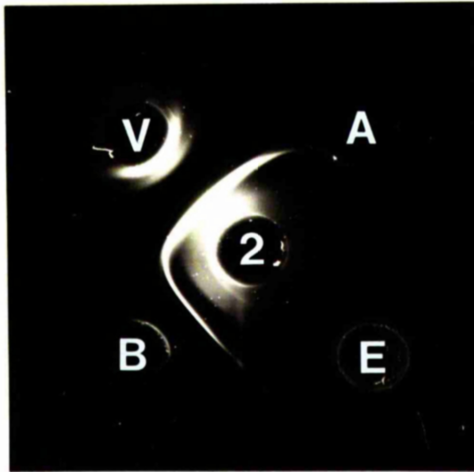

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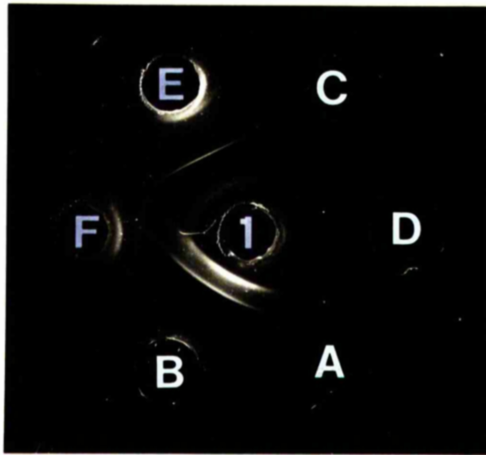

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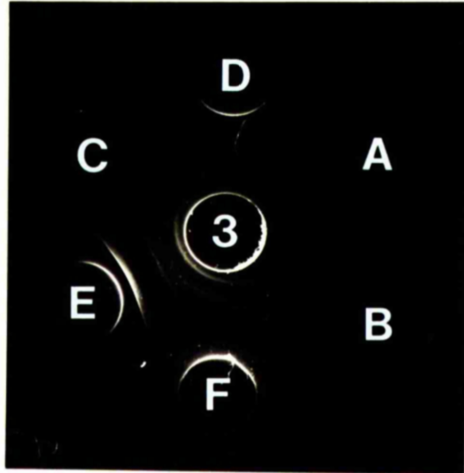

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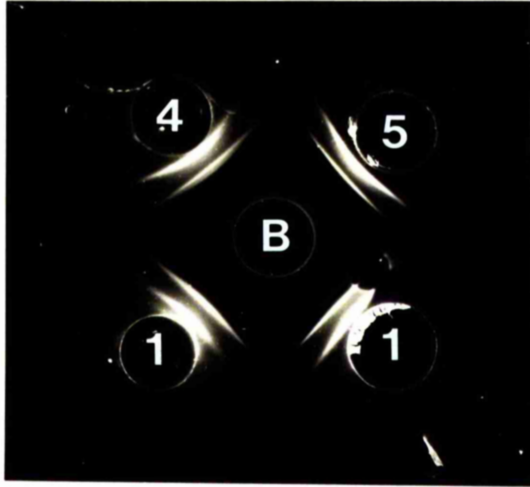

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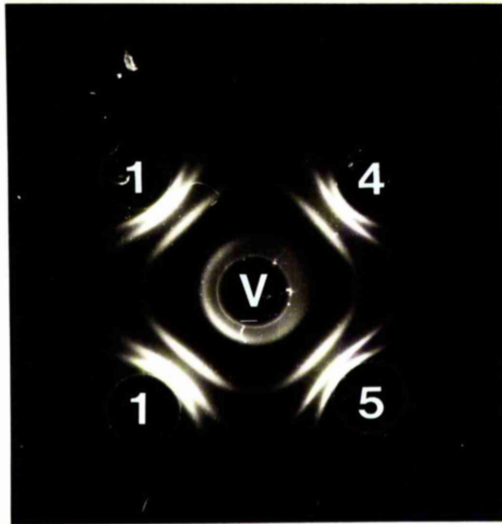

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DISCUSSION

The object of this work was to define the changes induced at the surface of host cells by either a productive or non-productive infection with vaccinia virus. In the case of many viruses, such foreign, virus specified antigens in the host cell membrane constitute the initial *in vivo* contact with the host immune system. Only recently has the importance of these antigens in eliciting the earliest of the immune responses to virus infection been realised (Porter, 1971; Notkins, 1974). However, for most viruses, the role of antibody to these antigens has still to be elucidated.

The strain of virus used in these experiments was originally derived from the Lister Institute strain (Westwood *et al*, 1966). Ito and Barron (1972b) and Koszinowski and Ertl (1976) examined similar Lister-derived strains and found minimal synthesis of early VICSA.

In the experiments described here the capacity for this derivative strain of Lister to induce early VICSA was unknown. In addition the antiserum used to detect surface antigens was, in most cases, rabbit hyperimmune serum to the same strain of virus. It was not known whether antibodies to VICSA were present in this antiserum.

However, since this work was carried out in the overall context of evaluating the nature of protection afforded by vaccination, the use of a derivative of the Lister Institute strain was justified as it has been shown to protect rabbits from challenge by rabbit pox virus (Boulter *et al*, 1964) and is an effective human vaccine for protection against smallpox. Consequently, any antigen not induced, particularly *in vivo*, is unlikely to be of importance in eliciting protective antibodies.

A. Early antigenic changes in the cell surface membrane of vaccinia-infected cells

Several methods were employed to detect new viral antigens appearing at the cell surface of unfixed monolayer cells and the results are briefly summarised in Table 28. Cell surface viral antigens were detected more efficiently using AIPM serum, which presumably contained a greater proportion of antibody directed against VICSA. The AIPM serum 35 showed consistently greater binding to virus-infected than to uninfected monolayers by immune haemadsorption, demonstrating that the specificity of the antiserum was directed mainly against virus-specific components of the plasma membrane. Antibodies to HeLa cell plasma membrane antigens were also present in AIPM serum 35 as judged by the slightly elevated immune haemadsorption to uninfected cells (Table 25). Immunofluorescence provided the most sensitive comparison of the various antisera (Plates 3 and 7). AntiVACγG detected VICSA as early as 2 h p.i. even when viral DNA synthesis was prevented. The AIPM serum, although staining uninfected HeLa cells to a low, background level (similar to that shown by AUPM serum), reacted strongly with virus-infected cells, and membrane fluorescence was evident from as early as 2 h p.i. in both productive and non-productive infection (where ara-C was present). From the nature of the immunising antigen, AIPM serum might be expected to contain antibody to at least one late cell surface viral antigen, VHA, and this was confirmed by the recorded HAI and HADI titres. Early binding (2 h p.i.) cannot be attributed to the presence on the cells of surface VHA, since other experiments failed to detect VHA at the cell

Table 28 Detection of VICSA on vaccinia-infected HeLa cells

a) antiVACγG

Technique	early (2 h p.i.)	late (24 h p.i.)	non-productive (no DNA replication)
Immunofluorescence	+	+	+
Immune haem-adsorption	+	+	n.t.
Complement fixation	±	±	n.t.
Complement-mediated cytolysis	±	+	n.t.
^{125}I-antibody binding	-	-	n.t.

b) AIPM serum

Technique	early (2 h p.i.)	late (24 h p.i.)	non-productive (no DNA replication)
Immunofluorescence	+	+	+
Immune haem-adsorption	+	+	n.t.

n.t. = not tested

"+" = surface antigen change detected

± = weak positive reaction

\- = no reaction

surface before at least 8 h p.i. However this does not exclude the possibility that a VHA precursor is an early VICSA. It is probable that AIPM serum contained antibodies to an early virus antigen or virus-modified host antigen at the infected cell surface.

Although antiVACγG gave only weak immunofluorescence against early VICSA, when the same serum was used in the immune haemadsorption assay for VICSA, antibody was readily bound to virus-infected cells from as early as 2 h p.i. (Table 25). The AIPM serum, however, was again more efficient in recognising VICSA, particularly late in infection, as evidenced by the substantial increase in binding by immune haemadsorption between 2 and 24 h p.i. Red cells adhered preferentially to vaccinia-infected cells both at 2 and 24 h p.i. suggesting that binding mediated by host plasma membrane components with their corresponding antibodies played only a minor role in this reaction.

The use of hyperimmune antiserum in VICSA assays by complement fixation and complement-mediated cytolysis, failed to reveal any major difference between 2 and 24 h p.i. In the case of fixation of complement, a depletion, presumably as a result of antigen-antibody reaction, was observed as early as 2 h p.i. but no significant increase was apparent by 24 h p.i. Cytolysis mediated by complement was more efficiently measured when antibody and complement were allowed simultaneously to interact with cell bound antigen. Using this technique only moderate differences were demonstrated at 2 and 5 h p.i. in complement-fixing VICSA using antiVACγG as compared to NγG, but the difference was significant by 24 h p.i. The relative insensitivity of the complement fixation assays may partially explain

the inability to detect VICSA in appreciable quantities. However only low levels of complement fixing antibodies may have been present in the γ-globulin concentrate or alternatively the anti-VICSA antibodies, if present, may not fix complement on interaction with VICSA. Although Ueda et al (1972) found that antiserum to early vaccinia soluble antigens was capable of fixing complement on reaction with extracts of infected cells, it was not established that complement was fixed when VICSA reacted with anti-VICSA.

There are several examples of antibodies which do not fix complement (reviewed by Mollison, 1969). Within the human blood group system, Rhesus (Rh) antibodies, which are predominantly of subclasses IgG_1 and IgG_3 do not bring about lysis of Rh positive erythrocytes. However of the four IgG subclasses only IgG_1 and IgG_3 bind well to complement component C_1q. It has been postulated that the paucity of Rh antigenic sites on the erythrocyte does not permit bridging of antibody molecules by C_1q (see Mollison, 1969). IgM is approximately 1000 times more effective than IgG in mediating erythrocyte lysis by complement (Humphrey and Dourmashkin, 1965) and it is likely that antiVACγG contains predominantly IgG antibodies, since IgM is associated mainly with the primary antibody response (Uhr and Finkelstein, 1967). Therefore the density of antigenic sites on the cell membrane and the class or subclass of antibody induced may affect the ability of cell-bound antigen-antibody complexes to fix complement.

The small changes both in depletion of complement and in dye uptake by cytolysis at 2 h p.i. may be due to residual virus or virion coat material (Chang and Metz, 1976) deposited on the cell

surface rather than VICSA. However a significant increase in dye uptake was demonstrable by 24 h p.i. This may be attributed to the presence of either early non-virion antigens at the cell surface supplemented by the emergence of late antigens or the movement of mature virions to cell peripheral locations. Among the late antigens appearing at the cell surface, VHA may account for the increased susceptibility of virus-infected cells to cytolysis although it is not clear whether anti-VHA may fix complement because of the difficulty in obtaining highly purified VHA.

Thus the Liverpool strain of vaccinia induced formation of virus-specified antigens at the cell surface from an early stage in infection and throughout the infection cycle. The facility of detection of these antigens varied according to the antiserum employed. AntiVACγG was less efficient in detecting VICSA than the rabbit AIPM antiserum despite the 128-fold greater HAI titre. Presumably there was a higher level of antibody to VICSA (both early and late) in the latter antiserum. An alternative explanation for the greater efficiency of AIPM serum in VICSA detection may lie in an "altered self" hypothesis originally postulated by Doherty and Zinkernagel (1975) and extended to vaccinia-infected mouse cells by Koszinowski and Ertl (1975b) where host cell surface antigens, particularly the transplantation antigens, may be modified. From later work with poxvirus-infected cells (Ada _et al_, 1976; Jackson _et al_, 1976) it was suggested that newly specified virus antigens interacted with existing host membrane antigens to produce an alien antigen determinant at the cell surface recognised both by the cellular and humoral components of the immune system. Therefore AIPM serum may contain antibodies to

altered components of the HeLa cell plasma membrane in addition to virus specified antigenic sites. Such antibodies directed against "altered HeLa" would be completely absent in antiVACγG raised against rabbit-passaged virus and would not be detected by AIPM sera 34 and 35 on vaccinia-infected RK13 cells.

Experiments designed to demonstrate the presence of viral antigen on the surface of vaccinia-infected cells by the double binding of rabbit antiVACγG and ^{125}I-SARγG were unsuccessful.

It may be assumed that VICSA was present on the surface of infected cells since this was demonstrable by other techniques - immunofluorescence, complement-mediated cytolysis and immune haem-adsorption - all of which depend on antibody-antigen interaction. The presence of late viral antigen, VHA, could be demonstrated on the cell surface by the binding of turkey erythrocytes. The m.o.i. used in the ^{125}I-SARγG experiments was sufficient to induce a high degree of HAD. The fact that VICSA was demonstrable by other techniques using the same rabbit antiVACγG suggested that the ^{125}I-SARγG, rather than antiVACγG may have been defective. Tests showed that the antigen-combining capacity of the SARγG was only moderately impaired by iodination and the antibody was still viable. However the low proportion of specific antibody in the labelled γG fraction compared to other protein may have resulted in a high background level of ^{125}I in the tests. Under these conditions, this method was not sufficiently sensitive to detect antigenic changes at the cell surface.

B. Late antigenic changes in the cell surface membrane of vaccinia-infected cells - vaccinia haemagglutinin

The time of appearance of VHA at the cell surface in the experiments reported here concurred with previous reports (Blackman and Bubel, 1972; Bubel and Blackman, 1975). The rise in infectious virus titre at 8 h p.i. preceded the rise in HAD titre by approximately 2 h but the temporal correlation between HAD and VHA reported by Blackman and Bubel (1972) was not found here. Whether this is significant in terms of the relationship between the two is doubtful as it probably reflects the varying sensitivities of the two assay techniques. The QHAD assay can detect small and smooth rises in titre whereas the HA test detects stepwise two-fold rises. The low m.o.i. employed here together with the small quantities of monolayer cells involved did not facilitate the detection of small rises in HA_{50} titre and it was only from 16 h p.i. that the large rise in HA_{50} titre could be measured. The sensitivity of the HAD assay is shown in experiments using varying m.o.i. where an m.o.i. as low as 0.01 still resulted in readily measurable HAD.

When changes in cellular or viral metabolism leading to the expression of VHA at the cell surface were investigated using various metabolic inhibitors, the reduction of viral DNA and protein synthesis resulted in a corresponding reduction in progeny virus yield, and the lack of HAD under these conditions confirmed the concept of VHA as a "late" antigen (Blackman and Bubel, 1972). Because of the low tolerance of HeLa cells to actinomycin D only reduced formation of VHA and mature virus occurred as demonstrated by Aasen and Haukenes (1972) using the same concentration of the drug.

Rifampicin, while arresting mature virus production completely, has been shown to allow a reduced but significant amount of VHA formation (Aasen and Haukenes, 1972), suggesting that the antigen is produced relatively independently of virus. Here VHA formation was reduced by 85 per cent and a background non-specific HAD to uninfected cells was apparent. However examination of individual, rounded virus-infected cells by phase contrast microscopy revealed that HAD did indeed occur. The almost complete absence of progeny virus however limited the formation of secondary foci. Similar examination of test plates, with other inhibitors, failed to yield any evidence for HAD positive primary foci.

The second section of the study of the role of VHA as a cell surface antigen was concerned with the interaction between VHA and red cells to establish the conditions under which binding occurred.

Erythrocyte binding to infected cells was optimal close to physiological pH and significant binding occurred over the pH range 5.75 - 8.70 (Table 10) in agreement with the findings of Chu (1948a) and Gurvin and Haukenes (1976) using haemagglutination titration.

The optimum incubation temperature, around 37°C, has also been reported by Nagler (1944) and McCarthy and Helbert (1960) by haemagglutination, and by Driessen and Greenham (1959) and Dekking and Van Dillen (1968) using haemadsorption. The substantial reduction in binding which occurred between 22.5°C and 7°C (Figure 4) could have arisen from the breakdown of normal lipid flow patterns leading to decreased mobility in the respective membranes. Alternatively as VHA

has been found to aggregate on storage at $4^{o}C$ (Chu, 1948a; Neff et al, 1965), aggregation or reorientation of VHA in the cell membrane may occur, thus reducing the capacity for red cell binding. Binding was stable at $58^{o}C$ for 30 min indicating that VHA is intrinsically heat stable, a finding previously reported (Chu, 1948a; Gillen et al, 1950; Gurvin and Haukenes, 1976). The heat stability of VHA in situ by HAD, has not however been previously reported.

From experiments on the relationship between HAD and the m.o.i. (Figure 3), the input virus determined the extent of HAD. However when different cell lines were infected with vaccinia at a constant m.o.i. progeny virus titres bore little relationship to the HAD found (Table 12). For a given cell line, however, the progeny virus titre may be related to HAD. The most extreme example tested, L-929 cells, with a similar virus output yielded 90 per cent less HAD than HeLa cells. No inherent inhibitor of VHA could be demonstrated in uninfected cell extracts. The formation of VHA may have been inhibited either at the transcription or translation stage of the protein moiety. Alternatively, a host component, possibly phospholipid, required for incorporation into VHA, may be present only at very low levels in L-929 cells. This may be phosphatidyl choline or phosphatidyl ethanolamine, both shown to participate with VHA-specific protein in reconstitution of viable VHA (Ichihashi, 1977), or, the selective removal of phospholipids for incorporation into VHA may result in a cell reaction to phospholipid deprivation leading to lipid rearrangement which may prevent expression of VHA at the cell surface. An analysis of cellular lipids and locations before and after vaccinia

infection of L-929 cells may elucidate this. The differing degrees of HAD shown in other cell lines may also be related to phospholipid levels. Since VHA-specific glycoproteins have been detected by PAGE then their absence from vaccinia-infected L-929 cells might implicate an inhibition of protein synthesis or glycosylation. It would be of interest also to determine whether extracts of vaccinia-infected L-929 cells react with anti-VHA antibodies.

One of the earliest observations on VHA was its specificity for agglutinating certain fowl red cells (Nagler, 1942). Of the eleven red cell species tested here, only turkey and mouse have been consistently shown to be agglutinable to any extent by VHA (Table 5). Neither cod nor African green monkey red cells have been previously tested while the agglutinability of cat and dog erythrocytes has also not been conclusively shown. All the red cell species displaying greater than 25 to 30 per cent HAD were found to be appreciably agglutinable (Table 11), and the results confirmed the non-adsorption of human, sheep and dog red cells (O'Connell et al, 1964). Erythrocytes of 50 per cent of Rhode Island red chickens may be susceptible to HAD (Dekking and van Dillen, 1968) but the red cells tested here seem to have originated from a negative bird. The results with turkey and mouse red cells confirm previous findings (Table 5), however the susceptibility of rat and rabbit red cells is not clear. The QHAD test gave a more sensitive assessment of red cell susceptibility than the HA test, and showed clearly positive HAD with rabbit and mouse erythrocytes and a slight positive reaction with rat red cells.

No destruction of VHA by trypsin was evident here in contrast

to previous findings (Blackman and Bubel, 1972; Gurvin and Haukenes, 1976). Chu (1948a) reported that VHA susceptible fowl erythrocytes were agglutinated following exposure to trypsin, but if trypsin did inactivate VHA in this test system and residual trypsin was responsible for any HAD, it would be expected that virus-infected and uninfected cells would show comparable adsorption of red cells. The differential binding of red cells to virus-infected cells was still evident however, at levels below 10 $\mu g\ ml^{-1}$ suggesting that minimal inactivation of VHA occurred, perhaps as a result of the inaccessibility of VHA to trypsin when *in situ* in the membrane.

Cells incubated during infection with varying concentrations of trypsin also displayed enhanced HAD at 24 h p.i., possibly attributable in part to trypsin adsorption of red cells. Normal cells treated with trypsin are rendered agglutinable by concanavalin A (Burger, 1969) and at certain stages of the cell cycle an increased agglutinability can also be observed (Fox, Sheppard and Burger, 1971). The increased agglutinability was originally thought to be due to exposure of con A receptors by trypsin; however it now appears that receptors are induced to cluster on the cell surface, probably by glycoprotein cross-linking, resulting in enhanced susceptibility to con A agglutination (Nicholson, 1972).

This dramatic effect of trypsin treatment on the surface membrane of normal cells illustrates the extensive restructuring of the cell surface which may occur on exposure to a proteolytic enzyme, and indicates that disruption of protein and lipid mobility may alter the capacity of the lipoprotein VHA to bind turkey red blood cells.

Trypsin may alter the cell surface in various ways; removal of mucopolysaccharides (Cook, Heard and Seaman, 1960), alterations of the distribution of receptor sites for both con A (see above) and anti-glycolipid antibodies (Hakomori, Teather and Andrews, 1968) and reduction in electrophoretic mobility (Barnard, Weiss and Ratcliff, 1969) have been reported. In addition it has been reported that after an initial lag phase (< 30 minutes) trypsin-dissociated cells regain the ability to aggregate (Steinberg, Armstrong and Granger, 1973). This latter observation may explain the haemagglutination reported by Chu (1948a).

Treatment of red cells with glutaraldehyde rapidly abolished their haemadsorptive qualities while prolonged exposure of infected HeLa cells to the fixative resulted in a progressive loss of the capacity to bind turkey red cells. Since glutaraldehyde is believed to cross-link protein molecules by reaction with free amino groups, producing cyclic and polymerised derivatives (Avrameas and Ternynck, 1969) either the red cell receptor is a protein or mobility of proteins in the membrane is necessary for HAD to occur. Haemagglutination tests with other viruses may be carried out with glutaraldehyde-fixed erythrocytes (Wolff _et al_, 1977) where the receptor is resistant to modification. This may indicate that VHA is an atypical viral haemagglutinin, binding red cells by an alternative mechanism to other HA-bearing viruses.

By the flotation method of Gurvin and Haukenes (1973), VHA could be appreciably purified from gross cell debris and the inhibitory effect of ara-C on the formation of VHA was confirmed by

these experiments. Gurvin and Haukenes (1973) claimed that this purification procedure produced material of high HA activity, which did not precipitate with antiviral serum in immunodiffusion tests and showed no evidence of polypeptide banding on PAGE. The complex banding pattern found here when "purified" VHA was subjected to PAGE casts doubt on the relative purity of VHA recovered by this simple purification procedure. Considering the low protein content of the purified VHA of Gurvin and Haukenes (10 $\mu g\ ml^{-1}$) it is perhaps not surprising that precipitin lines and polypeptide bands were undetectable. It might also be considered unlikely that such a simple procedure would result in substantial purification of one vaccinia antigen from what appears to be a highly complex mixture of viral antigens especially if associated with membrane fragments. In this study no immunodiffusion precipitin lines could be attributed to VHA. AntiVACγG, with an HAI_{50} titre of 1/16,000, detected no differences between antigen preparations before and after removal of VHA when these were compared by immunodiffusion. Therefore either the concentration of VHA in the antigen preparation was below the detectable limit or VHA was associated with material, possibly membrane fragments, unable to penetrate the immunodiffusion gel. If the latter explanation is correct then VHA/membrane fragments must be of considerable size as 3 per cent agar gels limit penetration of _Caminella_ haemocyanin (molecular weight 6.6×10^6) (Allison and Humphrey, 1960) and in this study 0.75 per cent agar was used.

The ability of hyperimmune antivaccinia serum to inhibit HAD of turkey red cells to vaccinia-infected cells was in agreement

with reported findings (Driessen and Greenham, 1959; Middelhoven, 1962). Haemagglutination of turkey red cells was also inhibited, but to a higher titre reflecting a difference in the relative sensitivities of HAI and HADI tests. The reverse is true in the case of HA and HAD assays where HA is a relatively insensitive measure of the VHA content of vaccinia-infected cells.

It is clear from this study and from other work that VHA is intimately associated with the plasma membrane and that 'soluble' VHA possibly consists of membrane fragments containing vaccinia specified components. Treatment of virus-infected cells with anti-vaccinia serum blocked both haemadsorption and haemagglutination by cell extracts with only slightly reduced yields of virus and antigens (Table 15b). The reduction in the HA_{50} titre must have resulted from a direct effect of antiserum on the infected cell surface.

The reappearance of VHA on the cell surface after removal of antiserum overlays could be attributed to formation of new VHA at the membrane rather than elution of bound antibody. Firstly, haem-adsorption in antiVACγG-treated cultures increased by a similar degree to cells treated with NγG over the 24 h period. It is of interest to note that in agreement with Oda (1963a), although virus yield was only 40 per cent of the control value, the HAD titre was more significantly reduced (25.5 per cent of control value) by 24 h after antiserum removal. Secondly, cycloheximide depressed the recovery in HAD titre noted in control cells after antiserum removal. This may not be related to direct interference with VHA formation but to the transport of VHA. If the surface is the origin of VHA (Blackman and

Bubel, 1972) then the inhibitory effect may be upon synthesis or transport of a VHA-precursor, possibly one or all of the glycoproteins described by Ichihashi (1977). Weintraub and Dales (1974) and Ichihashi (1977) correlated the HA activity of plasma membrane fractions with the appearance of virus-specified glycoproteins. These observations do not preclude the possibility that VHA may be intimately associated with the cell plasma membrane without having been derived from it. Stokes (1976) showed that vaccinia release from the infected cell proceeded by transfer from cytoplasmic factories to the tips of cell microvilli possibly along a cytoplasmic microfilament network. By electron microscopy, virus particles were found around the cell periphery enclosed in host cytoplasmic membranes. Although it has also been demonstrated, using ferritin-labelled antibody, that viral antigen becomes distributed over the entire cell surface except at the egress points of mature virus (Ichihashi et al, 1971), other observations (Blackman and Bubel, 1972; Bubel and Blackman, 1975; this study) indicate that VHA becomes detectable at the cell surface at approximately the same time as mature virus suggesting a possible relationship between the appearance of cell surface VHA and emerging virus. The movement of virus particles outwards from the factories may further affect the already disrupted host cell metabolism and the formation of a lipoprotein such as VHA may simply be a by-product.

Ichihashi and Dales (1971) failed to demonstrate an association between VHA in a soluble antigen extract and a membrane fraction (including plasma and cytoplasmic membranes) from uninfected cells. In this study, no in vitro association between VHA and purified plasma membranes from uninfected HeLa cells could be demonstrated.

This is compatible with the concept of VHA as a viral glycoprotein already associated with membrane components. The plasma membrane from vaccinia-infected cells may be premodified in some way to allow insertion of VHA precursor and it would therefore be of interest to examine possible interactions between plasma membrane and VHA-specific glycoproteins. This might answer the question of whether VHA originates at the plasma membrane (vide supra). Also, antibody treatment of monolayers with adsorbed erythrocytes elicited progressive detachment of bound red cells. That the elution was not passive is shown by the lack of effect of NγG indicating that the VHA/antibody binding affinity was much greater than the VHA/erythrocyte binding affinity.

Binding sites on the vaccinia-infected cell surface for susceptible fowl cells were distinct from those for con A. Some steric hindrance of turkey red cell binding was apparent but irrespective of this con A is bound to the cell surface at sites distinct from VHA and antiviral antibody has no effect on this union.

Antisera to host cell components inhibited HAD possibly because cell surface VHA may be partly composed of host material capable of reacting with such antisera or more likely because antibody/surface antigen interaction sterically hindered subsequent binding of turkey erythrocytes. It should be noted that antiserum raised to whole HeLa cells (AHeLa) and to a purified plasma membrane fraction (AUPM) were devoid of HAI activity suggesting that the above inhibition of HAD was a result of steric hindrance to red cell binding.

Antiserum raised to a plasma membrane fraction from vaccinia-

infected cells (AIPM) had significantly higher HAI_{50} and $HADI_{50}$ titres than AUPM sera. However, in view of the possible steric hindrance of anti-host cell sera discussed above, the HADI activity of AIPM serum may be partially ascribed to steric hindrance by anti-host components of the antiserum. Suitable cross absorption of AIPM sera by HeLa cells might remove such anti-host antibodies but antibodies to "altered-self" antigens of the host cell would not be removed by this treatment.

C. Further changes in HeLa cells during infection by vaccinia virus

Vaccinia-infected HEL cells became agglutinable by con A by 2 h p.i., prior to viral DNA replication, confirming the findings of Zarling and Tevethia (1971). In this virus-cell system it is not clear whether increased agglutinability of cells by con A reflects an increase in the number of lectin binding sites or a lectin-induced redistribution of existing sites into clusters. Cells transformed by oncogenic viruses show increased con A agglutinability apparently due to lectin-induced "capping", a clustering of existing receptors since transformed and normal cells seem to possess similar numbers of receptors (Nicholson, 1974).

Bubel and Blackman (1975) attempted to determine whether vaccinia infection did indeed induce an increase in lectin-binding sites in the cell surface. That this was the case, was suggested by the apparent increase in the number of mouse red cells bound to con A-treated vaccinia-infected cells and also by an increased binding of FITC-labelled con A.

The modified method of Bubel and Blackman described here, detected marked differences in the binding of con A-coated human erythrocytes but only comparatively late in the infection cycle. Superimposed on the fluctuating binding to virus-infected cells (Figure 11) was a background binding to normal HeLa cells. The fluctuation may be attributed to a variation in the number of con A receptors present on the surface of HeLa cells, a continuous cell line, which is the case with other transformed cells during the cell cycle (Fox *et al*, 1971; Smets, 1973) and particularly at mitosis (Glick and Buck, 1973). Some partial synchronisation of the cell culture may have occurred due to handling. Initial virus penetration provoked a sharp drop in con A-red cell binding followed by an equally sharp recovery. Differences in lectin binding between uninfected and vaccinia-infected HeLa cells can thus be observed from 14 h to 24 h p.i. to be completely consistent and reproducible.

From these findings, and from those of Zarling and Tevethia (1971) and Bubel and Blackman (1975), it can be concluded that vaccinia-infection produces a similar effect to transformation, by increasing the target cell agglutinability by con A, but differs from transformation by inducing an increase in the number of con A binding sites, demonstrable at certain times after infection.

Bubel and Blackman (1975) suggested that the binding sites for con A were distinct from VHA-red cell binding sites. As already discussed, phase contrast microscopy of vaccinia-infected HeLa cells treated with turkey cells and con A-human cells confirmed this. In contrast to the viral specificities exhibited by VHA, the lectin-binding

site was not neutralised by potent anti-viral γG, and neither was the "union" between receptor and con A-red cell broken by antiserum, as was the case with VHA/turkey red cells.

Con A receptors are believed to be glycoproteins (Nicholson, 1974). It might therefore be possible that the increased binding of con A shown by vaccinia-infected cells was mediated by the appearance of new viral glycoproteins at the cell surface. These indeed exist and include VHA components (Moss et al, 1971; Weintraub and Dales, 1974; Ichihashi, 1977). However, the lack of interaction of emerging receptors with anti-viral antibody suggests that these are of host origin and may represent a reaction of the host cell to virus infection. The state of the host cell metabolism may be a crucial factor in determining the allocation of lectin receptors at the cell surface as the results of the experiment with metabolic inhibitors suggest. It has been reported that the expression of con A receptors at the herpes virus-infected cell surface, measured by agglutination, requires viral protein synthesis (Tevethia et al, 1972). Inhibition of cellular protein synthesis in control cultures did not affect con A agglutinability but the authors also suggested that increased agglutinability was not a non-specific change caused by virus adsorption, penetration, or c.p.e. In contrast Zarling and Tevethia (1971) reported that increased cell agglutinability after vaccinia infection required early host protein synthesis, but not viral or host DNA synthesis. Poste (1972) proposed that lysosomal enzyme release at the cell surface during paramyxovirus infection was responsible for increased cell agglutinability by con A, in combination with a virus-induced modification of cell surface oligosaccharides.

The results obtained in this study suggest that with regard to con A-red cell binding the effects of metabolic inhibitors on the cell are greater than the effect of virus on the cell. The drastic effect of vaccinia infection on host metabolism, culminating in an eventual breakdown of host directed functions (Moss, 1968), may mimic the effect produced by the cell antimetabolites, actinomycin D and cycloheximide. The complete shutdown of host functions was originally attributed to a component of the invading virion which inhibited cellular protein synthesis (Moss, 1968). Recent studies (Bablanian, 1975) have suggested that early viral mRNA synthesis may be a prerequisite for this effect. Despite the relative inefficiency (c.f. cordycepin) of actinomycin D in inhibiting vaccinia mRNA synthesis (Metz and Esteban, 1972) there may be some correlation involving early viral mRNA synthesis, between the induction of c.p.e. and increased con A binding. The early viral mRNA may have a direct inhibitory effect on host protein synthesis (Bablanian, 1975). Thus the manifestation of new con A binding sites on the cell surface may represent a non-specific response of the cell to breakdown of protein and RNA synthesis.

The findings of Bubel and Lambert (1967) and Bubel and Blackman (1975) indicated that vaccinia-infected cells synthesised sialic acid in greater quantities than uninfected cells but the excess sialic acid probably had an intracytoplasmic location. In the experiments reported here vaccinia entry into the host cells initiated a sharp reduction in the sialic acid content of the cells. Only when mature virus appeared did the sialic acid content begin to rise.

Golubev et al (1971) reported that in vaccinia-infected cells late in infection total glucose catabolism increased but the proportion of glucose oxidised by the pentose phosphate cycle decreased while the rate of glycolysis increased. Coincident with the appearance of c.p.e., an alteration of cell membrane permeability was apparent, shown by leakage of glycolytic enzymes into the medium. Schümperli et al (1977) have also reported significant leakage of cytoplasmic enzymes from vaccinia-infected cells. One may speculate that the rate of sialic acid synthesis decreases partly because of such leakage of enzymes from cells. In addition, the late increase in glucose catabolism noted above may also be reflected in an overall increase in sialic acid synthesis, since this is intimately related to both glucose and phosphoenol pyruvate metabolism (Lehninger, 1970). Leakage of the sialic acid pool from cells may also be important. Cytopathic effect, and thus leakage of cytoplasmic enzymes, is not prevented apparently by the presence of ara-C and the depressed levels may be prevented from rising by the lack of late virus activity.

The drop in sialic acid content of infected cells also suggests that the auto-agglutinability of vaccinia-infected cells seen from 2 h p.i. (in con A agglutination experiment) is probably not due to the deposition of extracellular sialic acid.

D. HeLa cell plasma membranes: isolation, purification and characterisation and properties of antisera

An obvious means to study vaccinia-induced antigenic modifications at the cell surface is to examine the changes effected in the protein profile of the plasma membrane.

Purity of the plasma membrane fraction is essential for this approach. The plasma membrane fraction prepared here was devoid of "negative" enzymic markers for endoplasmic reticulum and lysosomal contamination and was enriched in "positive" enzymic markers. The specific uptake of ^{3}H-fucose by the plasma membrane fraction also suggested that the fraction was of high purity.

Studies on plasma membranes from vaccinia-infected cells have generally lacked adequate characterisation of the purity of the preparations. Wallach and Lin (1973), Oseroff, Robbins and Burger (1973) and Chang, Bennett and Cuatrecasas (1975) have all discussed at length the absolute necessity for using valid markers, where, for example, the presence of a certain enzyme in a cell is restricted to the plasma membrane. In HeLa cells, the Na^{+}, K^{+}-dependent ATPase is believed to be such a marker (Johnsen et al, 1974) and has been used as such during studies on the polypeptide profile of plasma membranes from vaccinia-infected cells (Weintraub and Dales, 1974). In other studies, plasma membrane fractions have been isolated and characterised with respect to 5'-nucleotidase (Blackman and Bubel, 1972; Jackson et al, 1976) although the specificity of this enzyme in terms of exclusive plasma membrane location has been questioned (Chang et al, 1975). It may vary in distribution according to the purification procedure adopted (Johnsen et al, 1974) and may fluctuate in activity during the cell cycle (Bosmann, 1970). In other studies on isolated plasma membranes from vaccinia-infected cells characterisation of the fraction obtained has been minimal (Bandlow et al, 1973; Ichihashi, 1977).

Polyacrylamide gel electrophoresis confirmed that the PM fraction was considerably less complex than the cell homogenate and several polypeptides associated with the 680 g pellet but not detectable in the 680 g supernate were probably associated with the nucleus (histones or non-histone chromatin protein). The fainter bands on polyacrylamide gels were not recorded satisfactorily by photography and substantial improvement in identification of membrane polypeptides might be obtained by autoradiography when using radio-labelled polypeptides.

Plasma membranes from vaccinia-infected cells differed little from the plasma membranes of uninfected cells except that three additional polypeptides were detected. The smallest polypeptide (≈ 34,000 daltons) was of a similar molecular weight to one of the three VHA-glycoproteins (12,000, 34,000 and 150,000) identified by Ichihashi (1977). A fourth polypeptide of a similar size to lysozyme, perhaps equivalent to the 12,000 daltons polypeptide described by Ichihashi (1977), may also be present in the PM fraction of infected cells. However, unlike the other three polypeptides, this was not consistently demonstrated. The 88,000 daltons polypeptide is unlikely to be one of the core associated virion polypeptides (Vp2a, 2b, 2c; Sarov and Joklik, 1972) but may be a non-virion polypeptide related to the early surface antigens.

The polypeptide of molecular weight approximately 56,000 daltons may be equivalent to the structural surface tubule polypeptide (57,000 to 58,000 daltons) described by Dales _et al_ (1976) and Stern and Dales (1976). The presence of this component in the PM fraction is compatible with the high levels of neutralising antibody to ICV induced on immunisation.

The failure of Jackson et al (1976) to demonstrate consistent differences in polypeptide composition of plasma membranes from vaccinia-infected and normal HeLa cells is probably a reflection of the insensitivity of the direct gel staining technique compared to autoradiographical detection of newly synthesised polypeptides (Weintraub and Dales, 1974).

As already discussed, isolated plasma membranes from vaccinia-infected cells and also from uninfected HeLa cells, were used mainly to raise antisera and the properties of these antisera compared with hyperimmune anti-vaccinia serum and anti-HeLa cell serum. In terms of potency, anti-HeLa serum reacted more strongly with uninfected HeLa cells by immunofluorescence and immune haemadsorption, than did antisera prepared to plasma membranes from either virus-infected or uninfected HeLa cells. Anti-HeLa and AUPM had significant and comparable HADI titres, presumably, as previously discussed, by steric inhibition of red cell binding, and zero HAI_{50} titres. AIPM 35 reacted strongly with virus-induced cell surface antigens, as measured by immunofluorescence and immune haemadsorption, and in these tests AIPM 35 exhibited greater activity against VICSA than antiVACγG. Although the plasma membranes were prepared from 24 h-infected cells, the positive immunofluorescent reaction seen on 2 h-infected cells is further evidence for a virus-specific antigen on the host cell prior to viral DNA synthesis. Further antigens are present at 24 h p.i. One of these "late" antigens is VHA; AIPM 35 and to a lesser extent AIPM 34 showed VHA-inhibitory activity to significant titres. This would confirm the likely presence of VHA as an integral part of the plasma membrane fraction of vaccinia-infected HeLa cells.

From the data on virus neutralisation, the most surprising finding was the marked difference in ND_{50} titres to ICV between AIPM 34 and 35. Although semi-purified, the plasma membrane fractions at 24 h p.i. would no doubt contain whole virus attached to membrane fragments but virus replication was prevented by pretreatment with UV light. It is of interest that despite the considerable difference in ND_{50} titre against ICV, only a minor difference in HAI_{50} titre is apparent. This confirms that anti-VHA antibody is unrelated to neutralising antibody to ICV.

It is not clear whether there is a correlation between anti-VHA and neutralising antibodies to ECV; comparison of a wider range of antisera to plasma membrane fractions would be required to determine this. Alternatively absorption of anti VHA antibodies by VHA-coated erythrocytes could be investigated. In a recent study, however, Tagaya et al (1977) failed to demonstrate any correlation between HAI_{50} titres and ICV or ECV neutralising titres in the sera of rabbits immunised with membrane fractions from vaccinia-infected cells (HA^+ or HA^-), heated virus or active virus. Preparations of early VICSA also failed to elicit neutralising or HAI antibody, but, as with the membrane fractions, skin resistance to live virus challenge was induced. These findings suggest that viral antigens other than VHA, present in cell membrane fractions from both HA^+ and HA^- vaccinia-infected cells and also on ECV may be important both in inducing a neutralising response and in inducing skin resistance. The VHA/ membrane fractions, which were not apparently characterised, were heated at 100°C for 30 min during preparation and such antigens may well have been destroyed by this procedure. Anti-ECV neutralising

antibody titres were very low ($\leqslant$ 1/512) compared to the values obtained by Appleyard *et al* (1971) (1/6,000; anti-rabbitpox) and in this study (1/16,000; anti-vaccinia)

E. Conclusions and general discussion

It is clear that poxviruses exert a profound effect on the host cell by virtue of complete metabolic reorganisation and, to a lesser extent, by modifications to the fine structure. Although the tubule polypeptide, VP4c, has been identified as the antigen responsible for elicitation of ICV-neutralising antibody (Stern and Dales, 1976), the prospect that ICV-neutralising antibody itself is not sufficient to prevent spread of ECV allows further speculation on the "neutralisable" antigens of ECV. The apparent inclusion of a membrane bound antigen, VHA, in the "envelope" of ECV (Payne and Norrby, 1976), with its implied derivation from the host cell plasma membrane, strengthens the assertion that virus-induced activity at the surface of vaccinia-infected cells may have some bearing on the immune response to poxvirus infection. This has been extensively demonstrated for the poxvirus altered H-2 cell surface antigens coexisting with new virus coded antigens in the mouse L-929 cell system where circulating T lymphocytes are activated to play an important role in early surveillance of virus-infected cells (Doherty *et al*, 1976).

Of the cell surface modifications investigated here, those of an antigenic nature are clearly immunologically important. From as early as 2 h p.i. VICSA can be demonstrated. The emergence of

this antigen(s) coincides with cell structural reorganisation, in the form of "rounding", a general decline in host metabolism, here typified by the drop in sialic acid content of the cell, increased con A agglutinability and slightly increased con A binding. The next major shift occurs as virions mature, when, simultaneously, more "late" antigens appear, the most easily measureable being VHA although others may exist.

In early reports on VHA, the antigen was considered to be "cytoplasmic" and "soluble" since high speed centrifugation failed to deposit appreciable quantities from cell sap (Chu, 1948a). Clear evidence for the existence of a "soluble" VHA is lacking; the technique for preparation of cell extracts containing VHA is normally so severe that membranes containing VHA would be fragmented. It is likely that all cellular VHA is membrane-bound; the inherent nature of the molecule suggests a close relationship with lipid-rich organelles, modified by the inclusion of a virus-specified protein(s). Evidence suggests the plasma membrane as the likely site of VHA "assembly", i.e. the combination of phospholipid and specific virus protein(s). However considering the highly complex network of interlinked membrane systems, it is possible that VHA may be formed on an intracellular membrane and rapidly transported to the plasma membrane. Its association with isolated plasma membranes from infected cells has been shown by Blackman and Bubel (1972) and in this study when surface VHA was "blocked" by antibody soluble VHA could not be detected in disrupted cells (Table 15b). Also uninfected plasma membranes do not themselves show any association with VHA *in vitro* suggesting that VHA does not exist free from membrane. Although

intracellular membranes represent a minor contaminant (by enzyme studies) of the plasma membrane fraction isolated here, VHA may be attached both to these and to the plasma membranes in this fraction. The mere presence of VHA in isolated plasma membrane fractions does not necessarily mean that the antigen originates here. Stokes (1976) has described the transport of virus via microfilaments to the cell periphery and it is possible that the host cytoplasmic membranes surrounding the emerging virion may be the site of origin of VHA, and the appearance of VHA at the cell surface may be related to the arrival there of mature virions.

Since neutralising antibody to ICV did not restrict spread of ECV (Boulter and Appleyard, 1973) and since VHA was detected on the envelope of ECV but not ICV, antibody to VHA might be of some importance in countering ECV spread. Vaccinia generally infects by cell-cell transfer (Smith and Sharp, 1960), only 10 per cent being released as ECV. Anti-VHA would be of little value in restricting virus spread in this fashion although the attachment of antibody to cell surface VHA may, from these experiments, depress overall virus production by restricting the formation of secondary foci. This may have some bearing _in vivo_. However some poxvirus strains may not produce HA (Cassel and Fater, 1958; Weintraub and Dales, 1974) and no strain of rabbitpox virus produces HA (Fenner, 1958). Virulence in the latter case may not be affected by the loss of VHA. The partial agglutination of erythrocyte species such as rabbit by VHA also requires investigation _in vivo_. The fate of ECV (containing VHA) shed from infected tissue _in vivo_ would then be questioned -

is ECV more easily "neutralised" or recognised by phagocytic cells when adhering to an erythrocyte? On the other hand the dissemination of ECV may be facilitated by erythrocyte adherence.

The question of the "protective" poxvirus antigen exclusive to ECV is still unresolved, if there is indeed such an antigen, but it is clear that virus-induced modifications to the host cell membrane structure and biochemistry have important implications for the host immune system and further investigation of the relationship between the infected cell membrane and ECV is required.

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APPENDICES

APPENDICES

1. BUFFERS AND DILUENTS

a) Tris(hydroxymethyl)aminomethane-HCl (Tris-HCl) buffers

Stock solutions (for final molarity of x/10)

A. 0.2 x M Tris

B. 0.2 x M HCl

50 ml of A + y ml of B diluted to 200 ml with H_2O

y	pH
5.0	9.0
8.1	8.8
12.2	8.6
16.5	8.4
21.9	8.2
26.8	8.0
32.5	7.8
38.4	7.6
41.4	7.4
44.2	7.2

Tris-HCl buffers were used at the following molarities and pH:

0.01M (10 mM)	pH 7.0
0.01M (10 mM)	pH 8.0
0.1M	pH 8.9
0.02M	pH 7.4

b) Tris-glycine buffer pH 8.3

0.6 g Tris + 2.88 g glycine (ammonia-free) made up to 1000 ml with H_2O.

c) Histidine monohydrochloride - Tris buffer pH 7.8 (0.06M)

Stock solutions:

A. 0.12M Tris (14.52 gl^{-1})

B. 0.12M Histidine monohydrochloride (25.16 gl^{-1})

25 ml of A + 15.2 ml of B diluted to 50 ml.

d) Glycine-NaOH buffer pH 8.6 (0.1M), pH 10.7 (0.4M)

Stock solutions:

A. 0.2M Glycine

B. 0.2M sodium hydroxide

25 ml of A + 2.0 ml of B diluted to 100 ml with H_2O

(0.1M, pH 8.6)

(0.4M, pH 10.7)

e) Veronal-buffered saline (VBS)

Sodium chloride 8.5 gl^{-1}; Barbitone 0.575 gl^{-1}; Sodium barbitone 0.20 gl^{-1}; Magnesium chloride 0.168 gl^{-1}; Calcium chloride 0.028 gl^{-1}.

f) "Saijo buffer" pH 7.2 (Saijo, 1973)

Tris 12.1 gl^{-1}; Sodium chloride 2.922 gl^{-1}; Magnesium chloride 0.102 gl^{-1}; Calcium chloride 0.001 gl^{-1}.

pH adjusted to 7.2 with HCl.

g) Sodium acetate buffer pH 5.0 (0.1M)

Stock solutions:

A. 0.2M sodium acetate (trihydrate)

B. 0.2M acetic acid

70 ml of A + 30 ml of B mixed.

h) Sodium phosphate buffers (Sorensen, 1909)

Stock solutions (for final molarity of x/10)

A. 0.2 x M sodium di-hydrogen orthophosphate

B. 0.2 x M di-sodium hydrogen orthophosphate (dodecahydrate)

y ml of A + z ml of B diluted to 200 ml with H_2O

y	z	pH
92.0	8.0	5.8
87.7	12.3	6.0
81.5	18.5	6.2
73.5	26.5	6.4
62.5	37.5	6.6
51.0	49.0	6.8
39.0	61.0	7.0
28.0	72.0	7.2
19.0	81.0	7.4
13.0	87.0	7.6
8.5	91.5	7.8
5.3	84.7	8.0

i) Phosphate buffered saline pH 7.4 (Dulbecco and Vogt, 1954)

(Dulbecco's PBS-A)

Calcium chloride (di-hydrate) 0.1325 gl^{-1}; potassium chloride 0.2 gl^{-1}; potassium dihydrogen orthophosphate 0.2 gl^{-1}; magnesium chloride 0.1 gl^{-1}; di-sodium hydrogen orthophosphate (anhydrous) 1.15 gl^{-1}; sodium chloride 8.0 gl^{-1}.

This was prepared by dissolving one PBS-A tablet (Oxoid Ltd.) in 100 ml of distilled water.

j) Citrate dextrose saline (CDS)

Dextrose 20.5 gl^{-1}; tri-sodium citrate 8.0 gl^{-1}; sodium chloride 4.0 gl^{-1}.

k) Chick erythrocyte diluent (CED) pH 6.8

The composition of this diluent, marketed by Gibco-Biocult Ltd., is given below. The solution was however prepared with the modifications listed:

Gibco-Biocult		Modifications
sodium chloride 8.766 gl^{-1}	:	sodium chloride 8.766 gl^{-1}
disodium hydrogen orthophosphate 11.795 gl^{-1} ($7.H_2O$)	:	disodium hydrogen orthophosphate (anhydrous) 6.246 gl^{-1}
sodium dihydrogen orthophosphate 21.526 gl^{-1} (H_2O)	:	sodium dihydrogen orthophosphate ($2.H_2O$) 24.337 gl^{-1}
calcium chloride 0.111 gl^{-1}	:	calcium chloride 0.111 gl^{-1}.

l) Löeffler's methylene blue

Saturated solution of methylene blue in alcohol 300 ml

Potassium hydroxide (0.01 per cent in H_2O) 1000 ml.

2. QUANTITATIVE SPECTROPHOTOMETRIC ASSAY OF RED CELL BINDING TO MONOLAYER CELL CULTURES:- DERIVATION OF THE EQUATION GIVING ABSOLUTE READINGS

To be determined: $\frac{E_{410} \text{ turkey red blood cells (T)}}{E_{280} \text{ HeLa cells (H)}}$ (or any given red blood cell species / or any given cell line)

Known $E_{410}H/E_{280}H = x$ .... (1) } both determinable and are fixed values for any given erythrocyte or tissue culture cell

$E_{410}T/E_{280}T = y$............ (2) }

E_{410} total readable value (R)/E_{280} total readable value (R):

measured directly by spectrophotometry of cell/erythrocyte lysates in saponin solution (0.005 per cent).

$E_{410}R = E_{410}H + E_{410}T$ assuming additivity .... (3)

$E_{280}R = E_{280}H + E_{280}T$ .... (4)

From (1) $E_{410}H = x.E_{280H}$

∴ in (3), $E_{410}T = E_{410}R - x.E_{280}H$ .... (5)

From (2) $E_{280}T = E_{410}T/y$

∴ in (4), $E_{280}H = E_{280}R - E_{410}T/y$ .... (6)

From (5) $E_{410}T = E_{410}R - x.E_{280}H$.

Substituting in (6) $E_{280}H = E_{280}R - \frac{1}{y}(E_{410}R - x.E_{280}H)$

$$= E_{280}R - \frac{1}{y}(E_{410}R) + \frac{x}{y}E_{280}H$$

$$\therefore E_{280}H - \frac{x}{y}E_{280}H = E_{280}R - \frac{1}{y}(E_{410}R)$$

$$\therefore E_{280}H\left(1 - \frac{x}{y}\right) = E_{280}R - \frac{1}{y}E_{410}R$$

$$\therefore E_{280}H = \frac{E_{280}R - \frac{1}{y}(E_{410}R)}{\left(1 - \frac{x}{y}\right)} \quad \ldots\ldots\ldots\ldots (7)$$

In (5) $E_{410}T = E_{410}R - x\,E_{280}H$

$$= E_{410}R - x(E_{280}R - E_{410}T/y) \qquad \text{(from (6)}$$

$$\therefore E_{410}T - x.E_{410}T/y = E_{410}R - x\,E_{280}R$$

$$\therefore E_{410}T\left(1 - \frac{x}{y}\right) = E_{410}R - x\,E_{280}R$$

$$\therefore E_{410}T = \frac{E_{410}R - x\,E_{280}R}{\left(1 - \frac{x}{y}\right)} \quad \ldots\ldots\ldots\ldots (8)$$

So from (7) and (8)

$$\frac{E_{410}T}{E_{280}H} = \frac{E_{410}R - x\,E_{280}R}{\left(1 - \frac{x}{y}\right)} \times \frac{\left(1 - \frac{x}{y}\right)}{E_{280}R - \frac{1}{y}E_{410}R}$$

$$= \frac{E_{410}R - x\,E_{280}R}{E_{280}R - \frac{1}{y}E_{410}R}$$

The final value of $E_{410}T/E_{280}H$ was computed using an Olivetti Programma 101 calculator.

3. STORAGE OF TURKEY ERYTHROCYTES

Since haemadsorption studies required regular supplies of turkey erythrocytes the optimum conditions for storage of turkey erythrocytes in liquid nitrogen were investigated. The procedure for storage of red blood cells by rapid freezing in liquid N_2 was adapted from that of Birkbeck, Chao and Arbuthnott (1974). Optimum storage conditions were determined using combinations of either dimethyl sulphoxide (DMSO) or glycerol as cryoprotective agent with each of three physiological saline solutions:

a) Chick erythrocyte diluent (CED)

b) Dulbecco's A phosphate buffered saline (PBS)

c) Citrate-dextrose saline (CDS).

Solutions of DMSO or glycerol in each diluent were prepared at concentrations from 0 to 40 per cent v/v. Aliquots of whole citrated blood (0.5 ml) were mixed with 0.5 ml aliquots of the test solutions in triplicate. The contents, in plastic 3 x 0.5" test tubes, were snap frozen by immersion in liquid nitrogen. After rapid thawing in a bath of tepid water, the red cells were diluted with 0.5 ml volumes of cryoprotective agent in diluent, at a concentration half that of the original test solution (i.e. the same concentration as the frozen red cell suspension). Intact red cells were collected by centrifugation and the supernatants removed, diluted and the $E^{1\ cm}_{410\ nm}$ recorded against a distilled water blank.

A standard haemolysate (100 per cent) was prepared by totally lysing an equivalent volume of blood with saponin and all E_{410} readings

were expressed as percentages of standard lysis and presented in graph form (Figure 17).

Glycerol was found to be a more efficient preservative than DMSO for turkey red blood cells and with PBS or CDS best protection was afforded by glycerol concentrations of 15 to 30 per cent. The third diluent, CED, was less useful in combination with glycerol except at the highest concentrations. When cryoprotective agent was omitted, least lysis was found in the case of PBS. Glycerol at a concentration of 15 per cent in PBS was found to be the best protective combination.

4. ESTIMATION OF TOTAL PROTEIN

This was carried out according to the method of Lowry *et al* (1951).

5. ESTIMATION OF TOTAL SIALIC ACID

The method of Warren (1959) was used as described in "Experimental Immunochemistry" E.A. Kabat and M.M. Mayer. 2nd Edition (C.C. Thomas, Illinois, U.S.A.), pp. 534 to 536.

Reagents

1. 0.2M sodium metaperiodate in 9M H_3PO_4.
2. 10 per cent sodium arsenite in 0.5M sodium sulphate/ 0.1N H_2SO_4.
3. 0.6 per cent thiobarbituric acid (fresh) in 0.5M sodium sulphate.
4. Cyclohexanone.

Figure 17. Determination of the optimum conditions for storage of turkey erythrocytes in liquid nitrogen

Employing three buffers and two recognised cryoprotective agents, and using differing concentrations of each agent in each buffer, the optimum concentration in the most efficient buffer for storage of turkey erythrocytes in nitrogen was determined. Red cells and cryoprotective agent/buffer were mixed and snap frozen in a liquid N_2 bath. The rate of thawing was standardised and the intact red cells centrifuged. Supernatants were harvested and the extent of haemolysis compared to a 100 per cent haemolysate of turkey erythrocytes in saponin solution.

Key:-

cryoprotective agent/buffer

○—○ glycerol/PBS

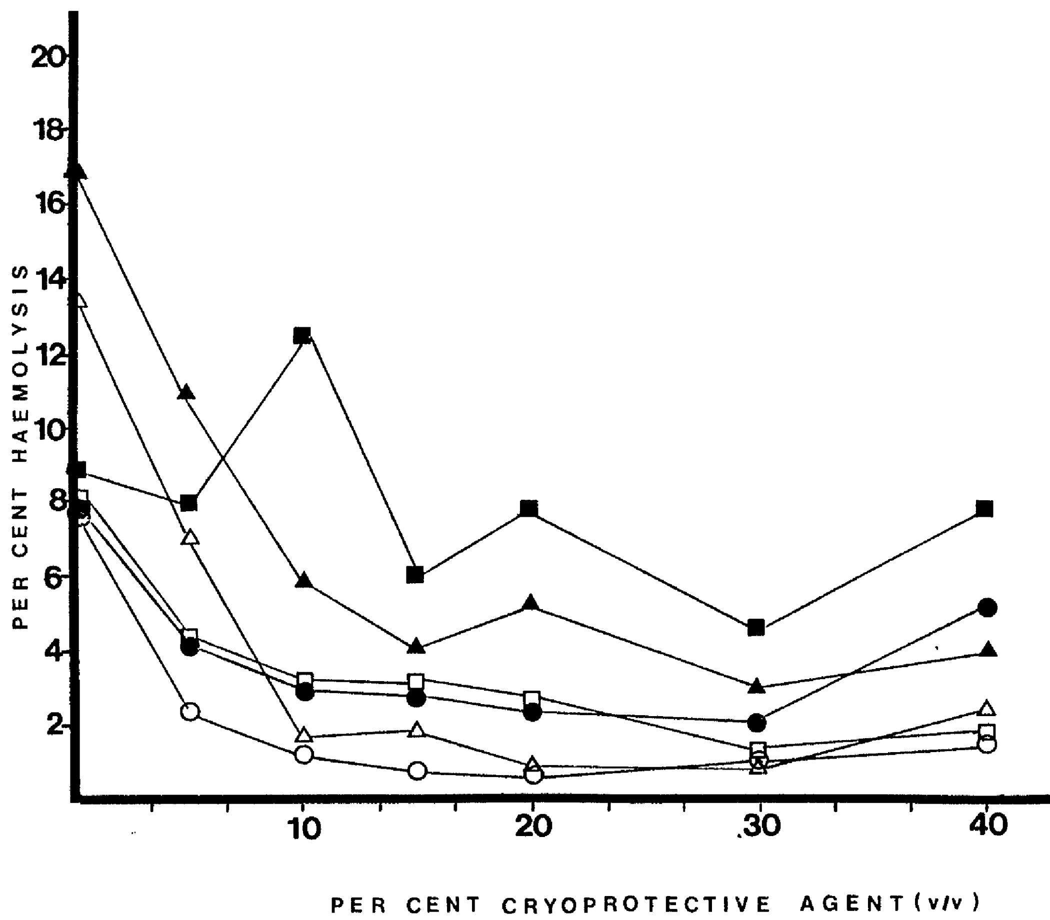
△—△ glycerol/CDS

□—□ glycerol/CED

●—● DMSO/PBS

▲—▲ DMSO/CDS

■—■ DMSO/CED


PER CENT HAEMOLYSIS
20
18
16
14
12
10
8
6
4
2
10
20
30
40
PER CENT CRYOPROTECTIVE AGENT (v/v)

Equal volumes of cell fractions (∿ 1.0 ml) and 0.2N H_2SO_4 in "Pyrex" conical centrifuge tubes were heated at $80^{o}C$ for 60 min and centrifuged at 1000 g for 15 min. The supernatants were decanted for assay of liberated sialic acid.

0.2 ml volumes of test supernatant were reacted in duplicate with 0.1 ml of reagent 1 by mixing and allowing to stand for 20 min. 1.0 ml of reagent 2 was then added and mixed until the yellow colour had disappeared. 3.0 ml of reagent 3 was added and the tubes incubated in a boiling water bath for 15 min, followed by immersion in cold water for 5 min. The pink-red colour was extracted with vigorous shaking by the addition of 4.3 ml of cyclohexanone. The tubes were centrifuged at 1000 g for 15 min and the upper layer removed for spectrophotometric determination. As recommended by Warren, a correction formula was used allowing for interference by 2-deoxyribose present in tissues, detectable by an absorption peak at 532 nm, in addition to the absorption peak of sialic acid at 549 nm.

Readings at 532 and 549 nm were taken (Pye Unicam SP500) and the molarity of the test sample with respect to sialic acid was determined, from the formula

$$\mu M \text{ N acetyl neuraminic acid}$$

$$= 0.09\, E^{1\text{ cm}}_{549\text{ nm}} - 0.033\, E^{1\text{ cm}}_{532\text{ nm}}$$

Standard assays containing 0, 5, 10, 15 μg (0 - 0.05 μM) N-acetyl neuraminic acid were also carried out.