ANTIGENS OF HERPES SIMPLEX VIRUS

A Thesis submitted to the

Faculty of Graduate Studies

University of Ottawa

In Partial Fulfillment of

the Requirements for the Degree

Doctor of Philosophy

by

Charlotte Rigby

August, 1971.

ABSTRACT

Herpes simplex virus (HSV) infects the majority of humans at some time in their lives; and this infection may persist for the lifetime of the host. The complex relationships between host and parasite which make such a long association possible are not yet understood.

The present study was undertaken in an attempt to clarify one aspect of that relationship, by characterizing some antigenic properties of HSV, of its host cell, and of the products of viral replication. The immediate objectives were threefold: first, to demonstrate the complete spectrum of antigens in HSV-infected cells; second, to characterize these as normal host antigens, as viral antigens, or as antigens with dual host-virus specificity; and, third, to distinguish between structural antigenic components of the virion and viral non-structural antigens involved in viral replication.

The antigens produced in cells of two unrelated host species (RK13 cells of rabbit origin, and F cells of chick embryo origin) as a result of infection with the H1 (type 1) strain of HSV were characterized, using a battery of rabbit and human analytical antisera. Soluble cell extract (SE) and purified virion (V) antigens were prepared from H1-infected cells of each host, and characterized by comparison, both with each other, and with comparable control antigens of uninfected host cells, using immunodiffusion,

neutralization blocking, and immuno-electron microscopy. SE antigens were further characterized by gel chromatography and acrylamide gel electrophoresis.

Eight antigenic components of SE antigens and two of purified virions were identified as viral antigens by their reactions with hyperimmune rabbit antisera. Two of these soluble antigens and one of the virion antigens also reacted with human convalescent sera, and were thus identified as major viral antigens, immunogenic in human infection, and common to more than one strain of HSV.

An internal virion antigenic component, released when purified virions were disrupted by treatment with sodium deoxycholate (DOC) reacted with hyperimmune antisera. Its presence in SE antigens was shown by immunodiffusion reactions.

Three unmodified host components of SE antigens, and two of purified virions were detected in viral antigens derived from Hl-infected F cells. No evidence was obtained by immuno-electron microscopy, or by neutralization blocking experiments to suggest that these unmodified host antigens were incorporated into virions; but the presence of a viral-modified host antigen in suspensions of purified Hl virions grown in RKl3 cells was suggested by its reaction with anti-host serum in immunodiffusion tests.

Gel chromatography on Sephadex G-200 resulted in the characterization of at least three viral antigens which eluted in the same protein peak. One of these was proven to be a viral structural antigenic component by immunodiffusion reactions with intact and DOC-disrupted virions. Neutralization blocking tests provided evidence that other structural antigenic components were also present.

Author- Charlotte Rigby

August, 1971.

ACKNOWLEDGMENTS

The author would like to express her gratitude to her fellow students, and to the staff of the Department of Microbiology and Immunology, University of Ottawa for their helpful advice and encouragement during the course of this work.

The assistance given by the members of the staff of the Animal House was invaluable.

The author was supported by a Studentship from the Medical Research Council of Canada.

This work was made possible through a Research grant from the Medical Research Council of Canada.

TABLE OF CONTENTS

continued....

TABLE OF CONTENTS (continued)

continued...

TABLE OF CONTENTS (continued)

IV. EXPERIMENTAL RESULTS

continued....

TABLE OF CONTENTS (continued)

LIST OF FIGURES

LIST OF TABLES

LIST OF PLATES

continued...

LIST OF PLATES (continued)

KEY TO ABBREVIATIONS

1. ANTIGENS

F antigens:	derived from F (primary chick embryo fibroblast) cells
$F\text{-}C_2$	F cell antigens of culture medium, concentrated approximately x500 by differential centrifugation
F-ex	extracted antigens of F cells, concentrated by centrifugation
F/SE	soluble antigens extracted from F cells
R antigens:	derived from RK13 cells (rabbit kidney cells)
$R\text{-}C_2$	RK13 cell antigens of culture medium, concentrated approximately x500 by differential centrifugation
R-ex	extracted antigens of RK13 cells, concentrated by centrifugation
R/SE	soluble antigens extracted from RK13 cells
H-F antigens:	derived from H1-infected F cells
$H\text{-}FC_2$	H1 virions in culture medium, concentrated approximately x500 by differential centrifugation
H-F/V	H1 virions purified by sucrose density gradient centrifugation after differential centrifugation
H-F/SE	soluble antigens of H1-infected F cells
H-F-ex	extracted antigens of H1-infected F cells, concentrated by centrifugation
H-R antigens:	derived from H1-infected RK13 cells
$H\text{-}RC_2$	H1 virions in culture medium, concentrated approximately x500 by differential centrifugation
H-R/V	H1 virions purified by sucrose density gradient centrifugation after differential centrifugation
H-R/SE	soluble antigens of H1-infected RK13 cells
H-R-ex	extracted antigens of H1-infected RK13 cells, concentrated by centrifugation

SE Antigens	soluble antigens, derived from lyophilized centrifuged extracts of H1-infected or uninfected RK13 or F cells
V Antigens	sucrose density-gradient-purified H1 virus suspensions

2. ANTISERA

HuH	human convalescent serum, collected from the author after an episode of recurrent herpes labialis
HuGG	pooled human immune globulin
NRS	normal rabbit serum
Ra_	prefix denoting Rabbit antiserum
RaF	hyperimmune rabbit serum, prepared against F-C_2, concentrated host antigens of F cells
RaHco	convalescent rabbit serum, collected after infection with H1 virus
RaHF-S	hyperimmune rabbit serum, prepared against H-F/SE, soluble antigens extracted from H1-infected F cells
RaHF-V	hyperimmune rabbit serum, prepared against H-F/V, purified virions grown in F cells
RaHR-S	hyperimmune rabbit serum, prepared against H-R/SE, soluble antigens extracted from H1-infected RK13 cells
RaHR-V	hyperimmune rabbit serum, prepared against H-R/V, purified virions grown in RK13 cells
RaR	hyperimmune rabbit serum prepared against R-C_2, concentrated host antigens of RK13 cells

3. OTHER ABBREVIATIONS

DOC	Sodium deoxycholate
ETH	ether
F-C_1	F cell host components in medium, after first stage of concentration procedure; approximately x50

H1	H1 strain of HSV, isolated by the author from a vesicle of recurrent herpes labialis; identified as HSV type 1
$H\text{-}FC_1$	F cell-grown H1 virions in medium, after first stage of concentration procedure; approximately x50
$H\text{-}RC_1$	RK13 cell-grown H1 virions in medium, after first stage of concentration procedure; approximately x50
HSV	herpes simplex virus
M199	medium 199 for cell culture
NBI	neutralization blocking index
PPLO	Mycoplasma
PSL	polystyrene latex spheres
PTA	phosphotungstic acid, 3%, pH 6.0; containing 0.05% bovine serum albumin
$R\text{-}C_1$	RK13 host cell components in medium, after first stage of concentration procedure; approximately x50
SDS	sodium dodecyl sulfate
TRY	trypsin

GLOSSARY

Analytical Antiserum	Serum containing antibodies produced in response to artificial immunization with host or viral antigens of known source and characteristics, or in response to natural or artificial HSV infection, which is used to detect and identify host or viral antigens in samples obtained by various preparative or fractionation procedures.
Antigenic Components	Individual antigens present in samples containing a mixture of antigens.
Control Antigens	Sedimentable components in the medium of control uninfected F or RK13 cells, concentrated x500 by centrifugation in the same manner as viral antigens.
Convalescent Sera	Sera collected 1-3 weeks after clinical HSV infection, confirmed by viral isolation.
Disrupted Virions	Virions of HSV broken into component parts by the action of chemical agents, such as enzymes or detergents.
Heterologous	This term is used in the context of this work to indicate an antiserum prepared against antigens derived from a host species different from that of the antigen being tested.
Homologous	This term is used in the context of this work to indicate an antiserum prepared against antigens derived from cells of the same host species as that of the antigen being tested.
Host Antigens	Antigenic components detectable in preparations derived from uninfected cells; or detected by antisera prepared against antigens derived from uninfected cells.
Major Viral Antigens	Viral antigenic components which react with several or all analytical antisera used.
Non-structural Antigen	An antigenic component produced during viral replication which cannot be shown to be identical to either antigens of the host or to structural antigenic components incorporated into the virion.

Nucleocapsid	The unenveloped virion of HSV.
Purified Virions	HSV suspensions after concentration and purification by differential and sucrose density gradient centrifugation.
Reaction of Identity	The reaction when two immunologically identical antigens react with antibody, or when two antibodies react with the same antigen in immunodiffusion tests. The precipitin lines formed by each identical component fuse, and a continuous precipitin line is formed between the respective wells.
Reaction of Partial Identity	The precipitin lines produced when two partially-related antigens react with a single antiserum (or vice versa) in immunodiffusion; indicated by spur formation, or by apparent splitting of the preciptin line.
SE Antigens	Soluble antigens of Hl-infected or uninfected RKl3 or F cells, derived from lyophilized cell extracts.
Soluble Antigens	Those antigens which remain in the supernatant after removal of virions from a suspension by ultracentrifugation.
Structural Antigens	Antigenic components incorporated into the virion; they may not be detectable in the intact virion by immunodiffusion.
Surface Antigens	Antigenic components of the surface of the intact virion; they are detectable in the intact virion by immunodiffusion.
Virion Antigens	Purified HSV suspensions.

I. INTRODUCTION

I. INTRODUCTION

Herpes simplex virus (HSV) is one of the most successful of human parasites. A majority of humans are infected at some time in their lives, but few serious illnesses are produced as a result of this infection; and infection, once established, may persist for the lifetime of the host, with little apparent damage to either host or parasite. The complex relationships between host and parasite, and the characteristics of each which make such a long association possible, are not yet understood.

The changes which occur in virus-infected cells reflect the nature of the virus, the nature of the cell, and the nature of the products of the interaction between them. The present study was undertaken in an attempt to clarify one aspect of that relationship, by characterizing some antigenic properties of HSV, of its host cell, and of the products of viral replication.

The immediate objectives, then, were threefold: a) to detect the complete antigenic spectrum of HSV-infected cells; b) to characterize the antigens detected as i) normal antigens of the host; ii) viral antigens; or iii) members of a newly-proposed class of antigenic determinants (Johnson & Westwood 1971), possessing dual host-viral specificity; and c) to further characterize the viral antigens as i) structural components of the

intact virion, or ii) non-structural viral antigens, including the enzymes which direct viral replication, and have been suggested as being important in HSV chronic infection and possible oncogenicity. (Tarro & Sabin, 1970).

The antigens of HSV, or of any member of the herpesvirus group, have not yet been characterized, either morphologically, as have antigens of the adenovirus (Ginsberg et al, 1966) or functionally, as have some of the antigens of the influenza virus (Scholtissek et al, 1969.) There are three main reasons:

First, the DNA of HSV is a large, double-stranded molecule. Its molecular weight, of around 100×10^6 daltons (Becker et al, 1968) has been calculated to contain enough genetic information to code for 250 average-sized proteins of molecular weight around 20,000 daltons (Fenner, 1970). Hence, the possible number of antigens is likely to be very large.

Secondly, antisera obtained from different sources, or produced in different ways, may react with different viral antigens, and this has given rise to some confusion in the literature. Thus Shipkey et al (1967) using fluorescein-conjugated hyperimmune rabbit antiserum, reported that HSV antigens were found only in the nucleus of infected HEp2 cells; Ross et al (1968), who also used immunofluorescence, showed that their hyperimmune rabbit antiserum reacted with viral antigens in both nucleus and cytoplasm of HSV-infected BHK cells; while the immunofluorescent studies of Roizman et al (1967) showed

that their hyperimmune rabbit antiserum reacted only with viral antigens located in the nucleus of infected HEp2 cells, while pooled human immune globulin reacted only with viral antigens in the cytoplasm. More recently, it was suggested that rabbits immunized with larger doses of HSV produce antisera reacting with a broader spectrum of antigens (Peutherer, 1970); and it has been reported that ferritin-labelled 7S or 19S antibodies of early (7-day) or late (7-week) antisera from the same animal reacted with different components of HSV as seen by electron microscopy (Miyamoto et al, 1971).

Thirdly, it was assumed until 1967 that only one serotype of HSV existed. The recognition of two distinct serotypes (Dowdle et al, 1967) was accompanied by the association of HSV type 1 with facial lesions, and HSV type 2 with genital infection (Nahmias and Dowdle, 1968), and followed by the recognition that these two serotypes differ in other characteristics as well, such as the type of CPE induced in infected cell cultures, (Kleger and Prier, 1969), virulence for laboratory animals (Plummer et al,1970), and mode of release from infected cells (Schwartz and Roizman, 1969). Some of the contradictions in the earlier literature are, no doubt, due to confusion between these newly-recognized distinct serotypes of HSV.

In recent years, interest in the herpesviruses has grown, as a result of evidence suggesting that HSV type 2 is associated with carcinoma of the cervix (Josey et al, 1968; Rawls et al, 1968;

Rawls et al, 1969), and of evidence associating herpes-like viruses with Burkitt's lymphoma (Stewart, 1969) and with infectious mononucleosis (Henle et al, 1968). The unique ability of HSV to cause chronic recurring infection in the face of high levels of serum neutralizing antibody has never been explained; similar mechanisms may be involved.

In view of these current problems, the antigenic characterization of HSV is a matter of some importance.

The present study was designed to characterize the complete spectrum of viral and host antigens of HSV-infected cells, since the effect of the host cell on the immunological characteristics of HSV has not yet been clearly defined. In order to accomplish this objective, two antigenically-unrelated host cell systems were chosen: the RK13 cell line, derived from rabbit kidney, and 'F' cells, primary chick embryo fibroblasts. RK13 cells were chosen because it has been reported that rabbits immunized with extracts of HSV-infected RK13 cells produced antibodies only to viral antigens, and not to antigens of the host cells (Watson et al, 1966). Antisera produced in rabbits against antigens of HSV-infected or uninfected cells of each host system were used for comparative immunological analyses, as well as rabbit and human convalescent sera.

Immunodiffusion was chosen as the basic method for the comparison of these antigens. Virion and soluble antigens, prepared from HSV-infected cells of each host species, were

characterized and compared with each other, and with comparable antigens of uninfected host cells, in order to identify viral and host antigens.

The use of agents such as trypsin, ether, or detergents to disrupt viral particles and release internal structural antigens not detectable in intact virions has proven to be a profitable approach in characterizing the structural antigens of other viruses. Westwood et al (1965) used this approach to show that seven of the soluble antigens of vaccinia virus were identical to those detected in trypsin-disrupted virions, and hence could be identified as virus structural antigens. Attempts to characterize the antigens of HSV by such an approach have not yet been reported in the literature. Controlled disruption of HSV virions was therefore undertaken in this study, using ether, trypsin, sodium dodecyl sulfate (SDS) and sodium deoxycholate (DOC) as disrupting agents. The antigens of disrupted virions were compared with those of the intact virion, of soluble (SE) antigens, and of SE antigens fractionated by gel chromatography or acrylamide electrophoresis, as a means of identifying and characterizing soluble virus structural antigens.

II. REVIEW OF THE LITERATURE

REVIEW OF THE LITERATURE

1. Morphology and Assembly of HSV Virions

Herpes simplex virus (HSV), like all herpesviruses, is a large enveloped virion, with a well-defined icosahedral capsid, containing 162 capsomeres arranged around a DNA core. The diameter of the naked nucleocapsid is 100 nm; the enveloped virion measures 150-180 nm (Roizman, 1969).

During replication of HSV, viral DNA is synthesized and accumulates in the nucleus. Viral protein, on the other hand, is synthesized in the cytoplasm, and then migrates to the nucleus, where encapsidation takes place. The assembled nucleocapsid moves to the nuclear membrane, and becomes enveloped before or during its passage through the nuclear membrane into cytoplasmic vacuoles (Roizman, 1969).

2. Structural Components of HSV

Chemical analysis of HSV (Russell et al, 1963) demonstrated the presence of protein (70%), DNA (6.5%), phospholipid (22%), and carbohydrate (1.6%).

Acrylamide gel electrophoresis of SDS - and urea - disrupted HSV virions has proven a useful approach in the study of the structural components of the virion. Spear and Roizman (1968), using this approach to characterize the structural polypeptides of HSV grown in HEp2 cells pulse-labelled with radio-active leucine

during the viral growth cycle, were able to identify six major structural proteins of the virion. At least 25 new proteins were first detected in the cell cytoplasm; all but three could be chased into the cell nucleus, and thence into naked and enveloped nucleocapsids. Olshevsky and Becker (1970) identified seven major proteins of HSV virions by these techniques. Two proteins formed the viral capsid; two were viral core proteins; and three proteins, identified as glycoproteins by incorporation of labelled glucosamine, were associated with the viral envelope.

Amino acid-labelled HSV preparations that were very highly purified by pre-treatment with fluorocarbon, DNAase, and RNAase, followed by density gradient centrifugation and elution from a calcium phosphate column were subjected to acrylamide gel electrophoresis by Robinson and Watson (1971), and eight structural polypeptides were demonstrated. Since electron microscopy showed that less than one per cent of the virions were enveloped, they suggested that these were the proteins of the naked nucleocapsid.

A similar analysis (Asher et al, 1969) of HSV lipid components showed that BSC-1 cells pre-labelled with ^{3}H-choline yielded HSV containing cellular lipids. Labelled lipid was associated with enveloped virions, and, as well, with intranuclear viral particles which were not yet enveloped. Furthermore, since 60-70% of these intranuclear nucleocapsids were dissolved by DOC, as indicated by changes in their sedimentation profiles,

it was suggested that lipid was a component of the nucleocapsid, as well as of the viral envelope.

3. Antigens of the Nucleocapsid

At least one antigen of HSV strains, required for infectivity and therefore presumably located on the surface of the nucleocapsid, is common to all HSV strains. Thus, routine serologic diagnosis of HSV infection rests on the demonstration in patients' sera of neutralizing or complement-fixing antibody reacting with any laboratory strain of HSV (Tokumaru, 1969).

This antigen, or a second antigen, however, possesses a sufficient degree of type specificity to allow the serologic distinction of HSV into types 1 and 2 by various modifications of the neutralization test (Wheeler et al, 1969), by immunofluorescence (Nahmias et al, 1969), or by indirect hemagglutination tests (Fuccillo et al, 1970).

Evidence for two distinct capsid antigens has been provided by ferritin labelling of 7S and 19S fractions of early or late rabbit antisera (Miyamoto et al, 1971). Only early 7S and late 19S antibody fractions reacted with the surface antigens of naked intranuclear capsids. The results of blocking experiments indicated that each antibody fraction reacted with a different specific antigen.

4. Antigens of the Virus Envelope

The proportion of enveloped virions reported in suspensions of HSV varies widely, from 5-10% (Robinson and Watson, 1971) to over 90% (Watson and Wildy, 1963a). The results of several electron microscopic studies (reviewed by Darlington and Moss, 1969) show that the viral envelope is derived from intracellular membranes, primarily that of the nucleus. It presumably, therefore, contains host cell components. In an early study, Watson and Wildy (1963b) suggested that, since enveloped virions of HSV were agglutinated by antiserum to normal cells, but not by antiviral serum, the HSV envelope contained only host antigens. Ferritin labelling, however, has more recently shown that the nuclear membranes of infected cells, from which the envelope is formed, acquires viral antigens while maintaining host cell antigenic specificity (Nii et al, 1968).

It has been known for some time that HSV infection induces the formation of viral antigens which alter the infected cell surface. Immune cytolysis of infected cells has been shown to occur in the presence of antiviral antibody and complement (Roane and Roizman, 1964), and Watkins (1965) reported that sensitized sheep cells were adsorbed to the membranes of HSV-infected HeLa cells, and that this adsorption could be specifically inhibited by anti-viral serum.

Acrylamide gel electrophoresis of extracts of purified membranes prepared from HSV-infected HEp2 cells labelled with ^{14}C-glucosamine was used to show that, during HSV infection of HEp2 cells viral-specific proteins are inserted into cellular membranes, and the label may later be traced to purified virions (Spear and Roizman, 1970; Spear et al, 1970). Keller et al (1970) demonstrated by similar techniques that, while the glycoproteins were genetically determined by the virus, since their composition varied with the strain of infecting HSV, the extent of glycosylation was determined by the host cell, since the amount of label varied when the same strain (MP) was grown in VERO or HEp2 host cells.

The influence of both virus and host envelope components on the characteristics of the virion is shown by the variation in buoyant density in CsCl of different strains of HSV grown in the same host cell, and of the same strain grown in different host cells (Spear and Roizman, 1967). Roizman (1969) concluded that these results reflect variations in the structural components of the viral envelope, since strains differing in their buoyant density also differed in surface characteristics such as elution from calcium phosphate columns and the ability to induce polykaryocyte formation in infected cells.

These more recent findings show that the envelope of HSV is made up of both viral and host components, both of which contribute to the biological, physical, and immunological properties of the virion. The biochemical evidence which has

been so far presented suggests that the viral component is glycoprotein in nature, and the results of Olshevsky and Becker (1970) suggest that at least three distinct viral glycoprotein antigens may be identified in HSV envelopes. The lipid components of the membrane seem, on the basis of present knowledge, to be of host origin.

5. Soluble Antigens of HSV

Soluble antigens may be defined as those antigens which are not sedimented by conditions of centrifugation under which intact virus particles are pelletted. Such soluble antigens may include a) unassembled virus structural components, b) non-structural viral antigens; that is, viral-induced proteins which are not destined to be incorporated into virions, and c) host antigenic material.

The existence of soluble viral antigens associated with HSV has been known since Hayward's initial description (1950) of a heat-labile complement-fixing antigen present in infected allantoic fluid after removal of infectious HSV by ultracentrifugation. Since titers of complement-fixing antibody to this antigen paralleled neutralization titers of human and guinea pig antisera to HSV, he suggested that the same antigenic determinants were present in both soluble antigen and infectious virus. Wildy and Holden (1954) later confirmed these observations, and suggested that the soluble complement-fixing antigen(s) were virus structural precursor(s).

The first suggestion that these soluble antigens were actually a mixture of antigens, which included non-structural viral-induced proteins was made by Gold et al, (1963) who showed that soluble complement-fixing viral antigens in HSV-infected BHK cells appeared before infectious virus was produced, and increased at a different rate. The demonstration by Keir and Gold (1963) that the levels of two enzymes, DNA nucleotidyl-transferase and DNAase I were raised in HSV-infected cells prior to the appearance of infectious virus raised the possibility that these viral-induced enzymes were non-structural viral antigens. Increased levels of DNA polymerase, dCMP deaminase, deoxythymidine kinase and dTMP kinase have since been reported in HSV-infected cells (reviewed by Keir, 1968).

The thymidine kinase of HSV-infected cells has been most intensively studied; it differs from the thymidine kinase of normal cells and of vaccinia-infected cells in several properties, including its pH optimum, Vmax, and Km (Kit et al, 1967; Klemperer et al, 1967). While this evidence is suggestive that the viral genome codes for these enzymes, it is not conclusive. For example, the aspartate transcarbamylase of adenovirus-infected cells, even though it has a pH optimum, Vmax and Km different from those of the enzyme in uninfected cells, has been shown to be a host enzyme whose characteristics have been altered by a virus-induced release from feedback inhibition (Ginsberg, 1969). More convincing evidence

that the virus genome codes for the thymidine kinase of HSV-infected cells was provided by the isolation of mutants of HSV which induced thymidine kinase in mouse fibroblast cells lacking the enzyme, and of mutants of HSV which were unable to induce formation of thymidine kinase, even though infectious virions were produced during infection. (Dubbs and Kit, 1964).

Finally, the inhibition of thymidine kinase activity of HSV-infected BHK cells by antiserum prepared against extracts of HSV-infected RK13 cells but not by antiserum prepared against normal BHK cells (Buchan and Watson, 1969) has provided more direct evidence for the viral coding of this enzyme. Therefore, thymidine kinase, at least (and possibly the other enzymes associated with HSV replication) may be identified as a HSV-specified non-structural antigen.

Arginine is required for the replication of HSV (Becker et al, 1967). The use of arginine depletion was investigated by Courtney et al (1970) as a means of blocking the synthesis of soluble viral antigens reacting in immunofluorescent or complement fixation tests with rabbit antisera to HSV. The results showed that, even though the production of infectious virus progeny was markedly inhibited, the levels of soluble antigens were not very much affected. Such results suggest a promising new approach to the study of HSV soluble antigens.

Immunofluorescence was used in a study by Roizman et al (1967) to show that at least five different viral antigens may be identified in HSV-infected HEp2 cells. Three viral antigens, associated with discrete intranuclear or cytoplasmic granules seemed to consist of virus structural elements, since antibodies with which they reacted could be adsorbed from the antisera by intact virus particles; two antigens, detected as diffuse intranuclear and intracytoplasmic fluorescence, still reacted with adsorbed antiserum, and were considered to be viral nonstructural antigens.

However, although complement fixation and immunofluorescence are sensitive and versatile tests, both are unable to resolve mixtures of antigens unless the antisera employed are monospecific, or are made so by adsorption with suitable antigens. Methods more suitable for the study of the soluble antigens of HSV are those which allow detection and identification of both the structural and non-structural components now known to be present in soluble antigen preparations. Immunodiffusion and ferritin-labelling techniques have been found to be useful for the characterization of these antigens.

Immunodiffusion was used by Tokumaru (1965) to show that a total of seven viral antigens, including one virion-associated antigen and three major soluble antigens could be detected in HSV-infected rabbit kidney cells by pooled human immune globulin. He

characterized the three major soluble antigens by gel chromatography, density gradient analysis, and immunoelectrophoresis. Two of these were identified as structural virus components because they were able to block neutralization of infectious virus by specific antibody, and could elicit the formation of neutralizing antibody in guinea pigs. One of these structural antigens had an estimated molecular weight of 500,000 daltons, and he suggested that it consisted of capsomeres; the second, with a molecular weight of 80,000 daltons, was thought to consist of capsomere subunits. No relationship to viral particles, and no possible function, could be ascribed to the third antigen, whose molecular weight was estimated at 170,000.

In a later study, eighteen precipitin lines were produced when fresh extracts of HSV-infected rabbit kidney cells were tested by immunodiffusion against pooled human immune globulin (Tokumaru, 1970). Some of these antigens were very labile, and disappeared after storage at $4^{\circ}C$ for eighteen hours. The principal stable antigens were identified as structural components of the virion surface by neutralization blocking tests. Only two antigens were present for which no such relationship could be shown, and these were thought to be nonstructural viral antigens.

The difficulties encountered in the detection of the labile soluble antigens of HSV were detailed by Tarro and Sabin (1970) who, using complement fixation, succeeded in demonstrating

a labile non-virion HSV antigen, but only after exhaustive adsorption of homologous hyperimmune serum with aged extracts of infected cells had removed antibody to the more stable antigens.

Ten antigens were detected by immunodiffusion when extracts of HSV-infected RK13 or BHK cells were tested against rabbit hyperimmune sera (Watson et al, 1966; Klemperer et al, 1967), and 12 were reported by Ross et al (1968). These antigens appeared at different times during the infectious cycle; they were considered to be both structural and non-structural viral antigens. Further results, suggesting that one of these non-structural antigens may be a group antigen common to HSV, B virus, and pseudorabies virus were presented by Watson et al in 1967. These three viruses shared a common precipitating antigen, yet were not cross-neutralized by antiserum against pseudorabies or HSV. This common, presumably non-structural, antigen is not likely to be associated with thymidine kinase, since antiserum to pseudorabies virus-infected cells did not neutralize the activity of HSV-induced thymidine kinase (Buchan and Watson, 1969).

Separation of the soluble antigens of HSV-infected BHK21 cells by acrylamide gel electrophoresis has been described by Watson (1969). Multiple protein bands were seen in stained gels; and four separate antigens were detected when proteins eluted from sections of the gel were tested against hyperimmune rabbit antisera by immunodiffusion. Specific antiserum, produced to one of these

antigens (Watson and Wildy, 1969) contained neutralizing antibody. Hence, this antigen is a structural component, located on the surface of the virion.

6. Conclusions

It seems likely that, in the future, as many antigens of HSV will be identified as can be detected by the antisera produced; and the limiting factor of the studies so far reported has been the potency and specificity of the antiserum used. Some viral or viral-modified host proteins may not be immunogenic during infection of the natural human host, and therefore only detected as antigens by hyperimmune sera produced in another species of animal. The known incorporation of host components into the infectious virion of HSV makes the characterization of such antigens a difficult task; any procedures designed to yield highly-purified HSV virions may succeed simply in removing essential viral envelopes along with non-virion host impurities.

As many as eighteen soluble antigens of HSV have so far been identified by immunodiffusion. Since some of these are very labile, special methods will be needed for their characterization. Two of the more stable antigens have been tentatively identified as structural antigens (Tokumaru, 1970), and one antigen, characterized by acrylamide gel electrophoresis, has also been shown to be a structural virion component (Watson and Wildy, 1969). No identification of a precipitating antigen as a non-structural protein, such as thymidine kinase, has yet been reported.

III. MATERIALS AND METHODS

MATERIALS AND METHODS

1. ANTIGENS

1. Host Cells

i) RK13 cells, a continuous cell line originally derived from rabbit kidney (Beale et al, 1963) were kindly supplied by Dr. J. Furesz, Canadian Communicable Disease Centre, Ottawa.

ii) F cells were primary chick embryo fibroblasts, prepared from 14-day embryos by standard techniques (Hsiung, 1964).

2. Cell Culture Medium

Medium 199 (M199), fetal calf serum, and normal rabbit serum (NRS) were purchased from Grand Island Biological Co (Gibco), penicillin, streptomycin, and aureomycin from Nutritional Biochemical Corp. (NBCo), and trypsin 1:250 from Difco Ltd.

The growth medium for the propagation and maintenance of all cell cultures consisted of M199 supplemented with 10% fetal calf serum and contained 100 u/ml penicillin and 100 ug/ml streptomycin.

3. Cell Culture Methods

i) RK13 cells were propagated and maintained in 32-oz. glass Brockway bottles. They were monitored regularly for PPLO (Mycoplasma) contamination, using methods described in Appendix I. Early in the course of this work, a PPLO strain was isolated from an RK13 cell culture. Since such PPLO contamination could lead to serious misinterpretations of the results of immunological analyses, the prevention of possible future contamination by the use of

aureomycin, as suggested by Hayflick and Stanbridge (1967) was investigated, as described in Appendix II. The results showed that 50 ug/ml aureomycin in growth medium prevented the establishment of PPLO infection. Therefore, before being used for the production of host or viral antigens, all RK13 cell cultures were passaged twice in growth medium containing 50 ug/ml aureomycin.

ii) F cells were suspended in growth medium after trypsinization and seeded into 32-oz. glass bottles at 2-3 $x10^5$ cells/ml. Batches of 20-24 bottles were prepared weekly.

4. Herpes Simplex Virus (HSV)

The H1 strain of HSV was isolated by the author from a vesicle of recurrent herpes labialis. It produced a typical HSV CPE in RK13 cells, and was identified by Dr. A.E. Kelen, Canadian Communicable Disease Centre, Ottawa, as herpes simplex virus type 1.

5. Preparation of Stock H1 Virus

i) H1-R stock virus (of Rabbit origin), used for the production of H1 virus antigens in RK13 cells, was prepared by passaging the initial isolate four times in RK13 cells. Infected cell culture fluid after the fourth passage was stored at $-80^{\circ}C$.

ii) H1-F stock virus, for the production of H1 virus antigens in F cells (i.e. of chick host origin) was prepared by passaging H1 virus, after its initial isolation in RK13 cells, twice more in F cells. Infected cell culture fluid after the second passage was stored at $-80^{\circ}C$.

6. Batch Production of H1 Virus

a) Infection and Growth of H1 Virus:

i) RK13 cells: H-R viral antigens were produced in batches of 20-24 monolayer cultures of RK13 cells. One bottle was used for the preparation of inoculum virus. The monolayer was washed once with M199, and 1 ml H1-R stock virus introduced. After adsorption of the virus for one hour at room temperature, M199 containing 2% normal rabbit serum and 50 ug/ml aureomycin was added, and the culture incubated at 37° until viral CPE was almost complete (24-48 hours). The infected cells were scraped into the medium, and used as the inoculum for batch production of virus in the remaining bottles. The cells were washed once with M199, and each was inoculated with 2-3 ml infected cell suspension. After adsorption for one hour at room temperature, M199 with 2% normal rabbit serum and 50 ug/ml aureomycin was added, and the cultures incubated at 37°.

ii) F cells: H-F viral antigens were produced in batches of 20-24 monolayer cultures of F cells in exactly the same way, except that the medium for virus growth consisted of M199 containing 2% fetal calf serum, and 100 u penicillin and 100 ug streptomycin per ml.

b) Harvesting of Virus:

When viral CPE was well-advanced (18-36 hours) the infected cells were scraped from the glass into the medium, and medium and cells separated by centrifugation at 2000 rpm for 20 minutes. The deposited cells, from which SE (Soluble Extract) antigens were later prepared, were resuspended in 10 ml deionized H_2O, and frozen at -80°C. The

medium contained the virion antigens, released from infected cells.

c) Control (Uninfected) Cell Cultures:

Comparable batches of control uninfected cells were prepared in the same way, using exactly the same procedures outlined above, but substituting sterile M199 for the original infecting stock virus inoculum.

7. Preparation of Antigens

Procedures for the concentration and purification of antigens were based largely on centrifugation. They are shown schematically in Figure 1.

a) SE (Soluble Extract) Antigens.

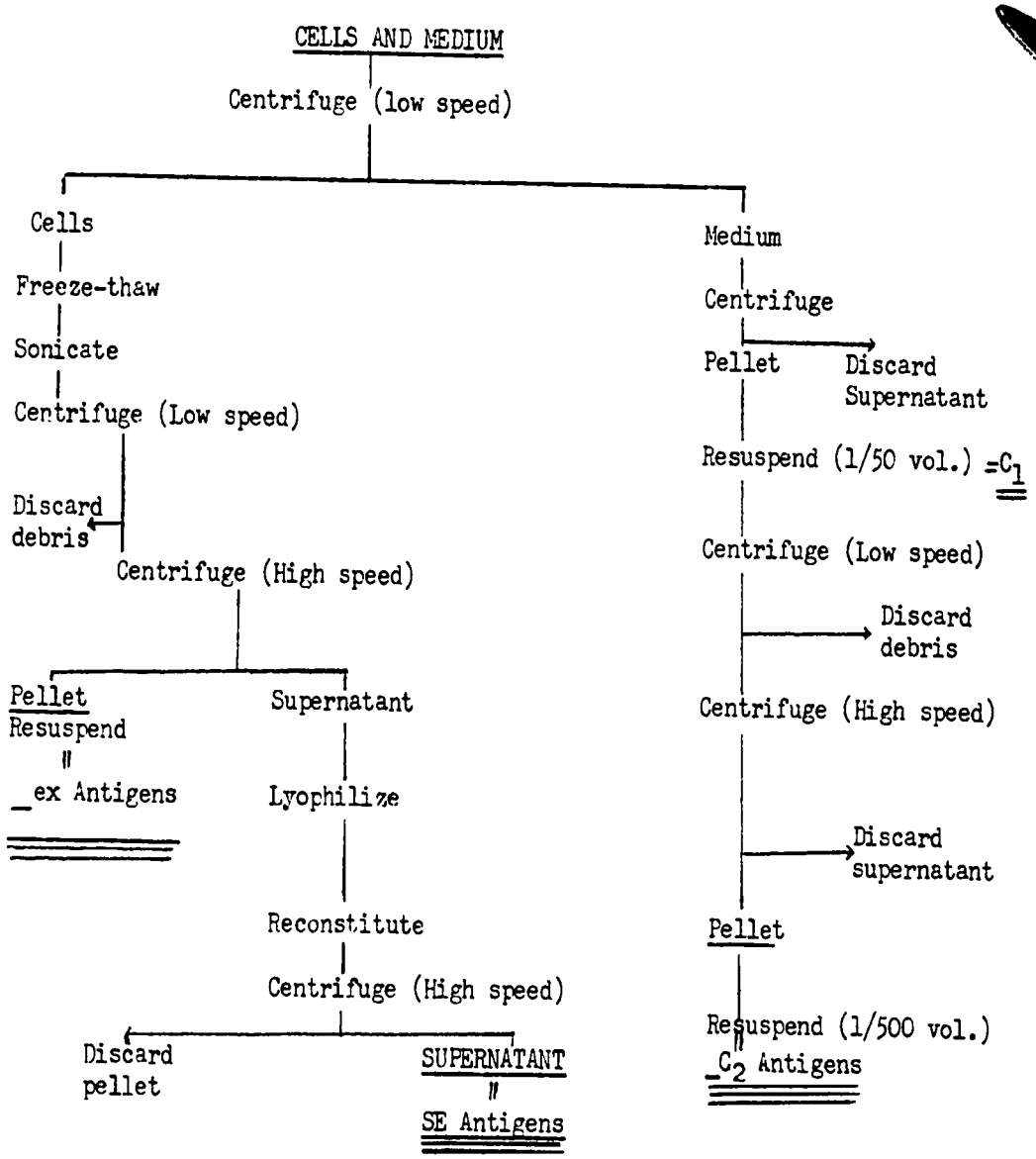
SE antigens were prepared from Hl-infected and uninfected cells of both RK13 (rabbit) and F (chick) host species. The cells, which were stored at -80°C, were thawed and further disrupted by sonication for two minutes at maximum frequency, using a Fisher-Blackstone Ultrasonic Generator. Virus particles and/or cellular debris were then removed by centrifugation at 100,000 g for sixty minutes in the type 50 rotor of a Beckman L-2 ultra-centrifuge, and the clear supernatant fluid was lyophilized.

The lyophilized antigens were weighed, reconstituted with deionized H_2O, and clarified by a second centrifugation at 100,000 g for sixty minutes.

The resulting SE antigens were designated by code letters indicating first, whether they were prepared from Hl-infected or uninfected cells, and second, their host derivation. Thus, H-R/SE

FIGURE I

FLOW CHART: PRODUCTION OF VIRAL AND HOST ANTIGENS



ANTIGENS

Antigen	RK13-derived		F cell-derived	
	viral	control	viral	control
Crude extract -ex	H-R-ex	R-ex	H-F-ex	F-ex
Soluble SE	H-R/SE	R/SE	H-F/SE	F/SE
Crude virion -C2	H-RC_2	R-C_2	H-FC_2	F-C_2

were soluble antigens of H1-infected RK13 cells, and H-F/SE were soluble antigens of H1-infected F cells, while R/SE and F/SE were soluble antigens of uninfected RK13 or F cells respectively.

b) Virion and Control ($-C_2$) Antigens

Preliminary experiments showed that H1 virus obtained by disruption of infected cells was accompanied by large amounts of host cell material which could not be removed by the usual procedures employed. Therefore, only the medium, from which infected cells had been removed by centrifugation at 2000 rpm for 20 minutes during the harvesting procedure was used as the source of virion antigens.

Infectious virions were pelletted from the clarified medium by centrifugation at 37,000 g for 150 minutes in a Backman L-2 ultracentrifuge, using the type 21 rotor. The pelletted virus was resuspended in about 20 ml saline to yield the first virus concentrate of the procedure: H-RC_1 if RK13 cell-derived; H-FC_1 if F cell-derived. After coarse debris was removed by centrifugation at 2000 rpm for 10 minutes, the virions were further concentrated by centrifugation at 100,000 g for 60 minutes, using the type 50 rotor of the ultracentrifuge. The pelletted virus was resuspended in about 4 ml saline to yield crude virion antigen concentrates: H-RC_2 if RK13 cell-derived, and H-FC_2 if F cell-derived.

Control antigens were prepared from uninfected cells of both host species in exactly the same way, and designated R-C2, if derived from uninfected RK13 cells, or F-C_2, if F cell-derived.

c) Purification of Virion Antigens H-RC_2 and H-FC_2

Sucrose density gradient centrifugation was used to purify crude concentrated virion antigens H-RC_2 and H-FC_2. Gradients were formed by layering 4 ml. amounts of ten sucrose solutions decreasing in concentration from 50% to 5% (v/v in H_2O) by 5% steps in tubes for the SW 25.2 Beckman rotor, and allowing the gradient to form 4-24 hours at 4°C. 2.5 ml. crude virion antigen H-RC_2 or H-FC_2 was then layered on the surface, and the tubes centrifuged 60 minutes at 18,000 rpm (40,000 g.) The opalescent band of concentrated virions was collected with a needle and syringe by puncturing the side of the tube, diluted to 20 ml. with deionized H_2O, and the virions pelletted by centrifugation in the type 50 rotor at 100,000 g for 60 minutes. The pelletted purified virus (virion antigen) was resuspended in deionized H_2O, designated H-R/V (if RK13-derived) or H-F/V (if F-derived), and stored at 4°C, since HSV has been shown to be more stable when stored in this way than at -80°C (Tokumaru, 1969).

d) Crude extract (-ex) Antigens

The material sedimented from extracts of Hl-infected or uninfected RK13 or F cells during the first high-speed centrifugation of the procedure followed for the preparation of SE antigens (page 21). was resuspended in 2-3 ml saline and designated crude extract antigen. These antigens were given abbreviations H-R-ex and H-F-ex for crude antigens of Hl-infected RK13 and F cells, respectively, and R-ex and F-ex for corresponding crude antigens of uninfected cells.

8. Antigen Assay Methods

a) Infectivity Assays

The infectivity of all viral antigens was assayed in RK13 cells, using plastic disposable 'Linbro' micro-titer plates (Winley-Morris Ltd.) and following a procedure modified from that described by Rosenbaum et al (1970). Serial tenfold dilutions of the virus suspension in 0.5 ml M199 were added to 0.5 ml growth medium containing 1 $x10^5$ RK13 cells, and 0.2 ml of the virus-cell mixture placed in each of four wells of the micro-titer plate. The plates were incubated at 37°C in a candle jar, and examined at 2, 4, and 6 days for viral CPE. Infectivity titers were calculated by the Reed-Muench method, and expressed as TCD_{50} per ml.

The sensitivity and reproducibility of this method were compared with those of infectivity titrations performed by the standard method of using pre-formed monolayers of RK13 cells in test tubes. The details and results of this comparison are given in Appendix III.

b) Protein Determination

The protein content of all antigen preparations was estimated by the Lowry technique (Leggett Bailey, 1962), using 'Labtrol' (Dade Reagents Ltd.) as the protein standard.

c) Electron Microscopy

i) Specimen preparation: Specimens were prepared for electron microscopy by placing a drop of the virus suspension on the surface of a carbon-stabilized formvar-coated copper grid,

adding a drop of phosphotungstic acid (PTA), adjusted to pH 6.0 and containing 0.05% bovine serum albumin, and, after a few seconds, draining the excess with filter paper. Stained grids were examined using a Philips EM 300 microscope; a Philips EM 100 microscope, made available through the kindness of Dr. J. Metuzals, was also used.

ii) Virus Particle Counts: Particle counts were obtained by a modification of the 'loop-drop' method of Watson and Wildy (1963). Polystyrene latex spheres (PSL) of mean diameter 126 ± 4 nm were purchased from the Dow Chemical Co. A standard 0.01% (w/v) suspension was prepared which contained 9.1×10^{10} spheres per ml, based on calculations according to the formula:

$$\text{No. PSL / gm dry weight} = \frac{1}{\text{vol. of sphere} \times \text{specific density}}$$

To 0.05 or 0.1 ml virus suspension was added an equal volume of standard PSL, and a drop of the mixture was stained and examined by electron microscopy. Virus particles and PSL spheres in representative fields of at least five different grid squares were tabulated, until a minimum of 100 PSL had been counted. Virions could easily be distinguished by their capsid structure at a screen magnification of 25,000.

The total number of virus particles was then calculated by direct ratio, according to the formula:

$$\text{Virions per ml} = \frac{\text{Total virus particles}}{\text{Total PSL spheres}} \times (9.1 \times 10^{10})$$

9. Disruption of H-R/V Purified Virions

SDS (sodium dodecyl sulfate, purchased from Fisher Scientific Co., and re-purified by the method of Roeppnack, 1965), DOC (sodium deoxycholate; Fisher purified grade), ether (Allied Chemicals Ltd.), and trypsin 1:250 (Difco Ltd.) were investigated as agents for the disruption of H-R/V purified virions (RK13 cell-grown).

a) SDS

To 0.8 ml H-R/V was added 0.1 ml 10% SDS in saline and 0.1 ml 1% B-mercaptoethanol (Matheson, Coleman and Bell, Ltd.). After incubation at 37°C for thirty minutes, with intermittent shaking, the preparation was dialyzed two hours at 37°C against saline containing 1% B-mercaptoethanol.

b) DOC

To 0.9 ml H-R/V was added 0.1 ml 10% DOC in saline, and the preparation incubated thirty minutes at 37°C, then dialyzed four hours against saline at room temperature.

c) Ether

An equal volume of ether was added to 0.9 ml H-R/V, and the mixture incubated thirty minutes at 37°C, with occasional vigorous shaking. The ether layer was then removed, and the residual ether evaporated using a gentle stream of nitrogen gas.

d) Trypsin

To 0.8 ml H-R/V was added 0.1 ml 1% trypsin, and the mixture incubated at 37°C. After sixty minutes, 0.1 ml 1% soybean trypsin inhibitor (NBC6) was added.

e) Control

Control untreated H-R/V antigens were prepared by adding 0.1 ml saline to 0.9 ml H-R/V and incubating thirty minutes at 37°C. Treated and control antigens were stored at 4°C.

10. Fractionation of SE Antigens

a) Gel Chromatography

SE antigens H-R/SE and H-F/SE (from Hl-infected RK13 or F cells) and R/SE and F/SE (from uninfected cells) were fractionated on Sephadex G-200 superfine (Pharmacia) in a 1.5 x 30 cm column prepared by the methods outlined by Curling (1970). Samples containing 8 mg protein in 1 ml 10% sucrose were applied to the top of the column, and Tris buffer (0.05M Tris plus 0.1% NaN_3, pH 7.2) allowed to flow through at 3 ml per hour. The void volume was 11.5 ml. Fractions were collected in 1.5 ml volumes with an LKB fraction collector, and the absorbance of each fraction determined with a Beckman DB spectrophotometer. Fractionated SE antigens were stored at 4°C.

b) Acrylamide Gel Disc Electrophoresis

The method described by Davis and Ornstein (1968) was followed for the electrophoresis of SE antigens H-R/SE and H-F/SE (from Hl-infected cells) and R/SE and F/SE (from uninfected cells) in acrylamide gels. Acrylamide solutions containing 7% acrylamide (Eastman), 0.18% Bis (N,N-methylenebisacrylamide) (Eastman), 4.6% Tris (Sigma), 0.02% TEMED (N,N,N',N'-tetramethylethylenediamine) (Eastman) and 0.07% ammonium persulfate (Fisher) (pH 8.9) were

polymerized in 5 x 75 mm glass tubes and pre-run 60 minutes at 1.5 mA/tube in a Buchler disc electrophoresis apparatus containing 0.005M Tris-glycine buffer pH 8.3. Samples containing 100 ug protein in 0.1 ml 20% sucrose were then layered on top of the gels, a trace of bromophenol blue tracker dye added to the buffer in the top chamber, and samples run into the gel at 1.0 mA/tube. Electrophoresis at 2.5 mA/tube was allowed to proceed about one hour, until the bromophenol blue band had migrated to approximately 1 cm from the bottom of the tube, and the gels were then removed from the tubes. The protein bands of some tubes were identified by fixing the gels in 12% trichloroacetic acid 30 minutes, staining with 0.25% aqueous coomassie blue 90 minutes at 37°C, and destaining and storing the gels in 10% acetic acid.

Three duplicate unstained gels were sliced into 5 mm segments, and the corresponding segments of each of the gels pooled. 0.3 ml 0.2M Tris buffer (pH 7.2) was added to the pooled gel slices, and protein eluted from the gels by shaking 48 hours at room temperature.

2. ANTISERA

1. Rabbit Antisera

a) Hyperimmune Sera

Antisera RaR and RaF, to control antigens R-C_2 and F-C_2 were prepared in four rabbits by the subcutaneous injection of 0.5 ml antigen (2 mg protein per ml) emulsified with 0.5 ml Freund's adjuvant (Difco). Three immunizing injections were given at one month intervals, the first containing complete adjuvant, and the second and third, incomplete adjuvant. Sera were collected two weeks after the last injection.

Antisera RaHR-V and RaHF-V, to purified virion antigens H-R/V (RK13 cell-grown) and H-F/V (F cell-grown) respectively, were each prepared in four rabbits immunized with 0.5 ml purified virions (0.2 mg protein per ml), emulsified with 0.5 ml Freund's adjuvant and inoculated intramuscularly. Four inoculations were given at monthly intervals, using complete adjuvant for the first and incomplete adjuvant for the later injections. Trial bleeds were taken, and when a high titer of neutralizing antibody was present, the animals were bled out, four weeks after the last inoculation.

Antisera RaHR-S and RaHF-S, to H-R/SE and H-F/SE soluble antigens of Hl-infected RK13 or F cells respectively, were each produced in two rabbits by the intramuscular inoculation of 0.5 ml SE antigen, containing 6 mg protein per ml, emulsified with 0.5 ml Freund's adjuvant. The first inoculation contained complete adjuvant, and the second and third, incomplete. Sera were collected three weeks after the last inoculation.

b) Convalescent Sera

Convalescent rabbit antisera RaHco were obtained from six rabbits which were infected with H1 virus by the intradermal inoculation of 0.1 ml virus suspension containing 10^7 TCD_{50} per ml into each of four sites in a shaven area on the animal's back, with the simultaneous injection of 0.5 ml intravenously. The establishment of viral infection was confirmed by the development of typical herpetic vesicles at the sites of intradermal inoculation, and by the isolation of infectious virus from vesicular fluid six days after infection. Sera were collected three weeks later.

Pre-immune sera were collected from all rabbits before the start of immunization schedules.

2. Human Sera

Human pooled immune globulin (HuGG) was purchased from Connaught Laboratories, Toronto. It was diluted 1:4 for use.

Human convalescent serum HuH was obtained from the author, from whom the H1 strain had been isolated, within a week of viral isolation.

All antisera were stored at -20°C, without preservatives and without prior inactivation.

3. Adsorption of Antisera

a) Adsorption of Rabbit Antisera with Fetal Calf Serum

Preliminary experiments showed that several rabbit antisera contained precipitins for the fetal calf serum used in growth

medium for both RK13 and F cells. Accordingly, all rabbit antisera were adsorbed with fetal calf serum.

One ml. aliquots of rabbit antisera were mixed with 0.1 ml fetal calf serum and incubated two hours at 37°C, then overnight at 4°C. The next day precipitates were removed by centrifugation at 1500 rpm for 15 minutes. The adsorption was repeated once, and the antisera were shown by immunodiffusion tests to be free of precipitins for fetal calf serum before they were used in analytical reactions.

b) Adsorption with Control Antigens R-ex or F-ex

One ml aliquots of selected antisera were mixed with 0.1 ml R-ex or F-ex (crude antigens of uninfected RK13 or F cells, respectively) and incubated two hours at 37°C, then overnight at 4°C. The next day, precipitates were removed by centrifugation at 2000 rpm for 20 minutes, and the adsorption was repeated once.

c) Adsorption with Virion Antigen H-R/V

Selected antisera were adsorbed with H1 virion antigens by mixing 0.45 ml serum with 0.05 ml H-R/V. After incubation at 37°C for two hours and overnight at 4°C, the sera were centrifuged at 2000 rpm 20 minutes. The adsorption was repeated twice.

3. IMMUNOLOGICAL TECHNIQUES

1. Neutralization

Doubling dilution of antiserum were prepared in 0.25 ml M199 in serological tubes, and mixed with 250 TCD_{50} H1 virus in 0.25 ml M199. After incubation for one hour at 37°C, 0.5 ml RK13 cells in growth medium ($1x10^5$ cells) were added to each tube, and 0.2 ml of the suspension seeded in each of four wells of a micro-titer plate. Plates were incubated at 37°C in a candle jar, and examined at 2, 4, and 6 days for the development of viral CPE. The neutralization titer of the serum was expressed as the reciprocal of the 50% serum endpoint dilution, calculated by the Reed-Muench formula.

2. Neutralization Blocking

Doubling dilutions of antiserum were made in 0.25 ml volumes of M199 in serological tubes and mixed with 0.25 ml of the antigen to be tested. After incubation at 37°C for sixty minutes, 0.25 ml H1 virus suspension containing 250 TCD_{50} was added, and the tubes incubated a further sixty minutes at 37°C. Approximately 1×10^5 RK13 cells in 0.25 ml growth medium were then added to each tube, mixed, and 0.2 ml aliquots of the mixture placed in each of four wells of a micro-titer plate. The plates were incubated at 37°C in a candle jar and observed at 2, 4, and 6 days for the development of viral CPE.

Control titrations included in each experiment were a) neutralizing activity of the antiserum pre-incubated with M199, and b) infectivity of the virus suspension. Neutralizing titers of

each serum-antigen mixture and of the control neutralization titration were calculated by the Reed-Muench method, and expressed as the reciprocal of the serum 50% endpoint dilution. The neutralization blocking index (NBI) of each antigen was then expressed by the formula:

$$NBI = \frac{\text{Titer of serum pre-incubated with antigen}}{\text{Titer of serum pre-incubated with M199}}$$

3. Immunodiffusion

Cellulose acetate immunodiffusion tests were carried out on microscope slides with the perspex templates described by Johnson et al (1964), using 'Serometrics' (Colab Ltd.) or 'Celotate' (Millipore Filter Co.) cellulose acetate strips and Tris buffer (0.2M, pH 7.2). Precipitin lines were allowed to develop 40-48 hours at 22°C.

Comparative immunodiffusions of some reactions were made in 1% agarose, using the perspex templates, according to the procedure outlined by Crowle (1958).

All immunodiffusion reactions were stained with thiazine red (Crowle, 1958), and direct enlargements made on F-3 Kodabromide paper (Eastman-Kodak Ltd.).

4. Immunoelectrophoresis

Attempts were made to characterize SE soluble viral antigens by standard immunoelectrophoretic methods, and are described in Appendix IV. Although fractionation of HSV antigens by immunoelectrophoresis has been described (Tokumaru, 1970), it did not prove to be of value in this study, and its use was not pursued further.

5. Immuno-electron Microscopy

Initial attempts to use immuno-electron microscopy for the visualizing of viral agglutination caused by specific antibody produced inconclusive results, since many H1 virus control suspensions also contained large clumps of virions, which could not be dispersed by sonication for as long as six minutes. Accordingly, the technique described below was developed in order to obtain an objective estimate of specific viral agglutination by immune sera. Virus suspensions were incubated with immune serum or with control NRS or pre-immune serum, PSL added, and specific agglutination of H1 virions measured by comparing the ratios of free virions to PSL in virus suspensions incubated with immune sera to those of the same virus suspension incubated with control serum, using the two-sample t-test.

Virus suspensions containing 10^{10}-10^{11} virus particles per ml were suitable for immuno-electron microscopy. 0.1 ml virus suspension was mixed with 0.1 ml serum diluted 1:10 in saline, and incubated at 37°C. After thirty minutes, 0.1 ml PSL was added and thoroughly mixed. Grids were prepared of each mixture, stained, and examined by electron microscopy as described on page 26.

The relative concentrations of free single virions and of PSL in each mixture was determined by counting the relative numbers of each in 21 different fields, chosen from at least six different grid squares, at a screen magnification of approximately 25,000. At least 200 PSL were counted in each sample. The ratios of free virions to PSL

in each field were determined, and the mean and variance of these ratios calculated for each serum-virus mixture. The values obtained for immune sera were compared with the values obtained for pre-immune or NRS controls by the two-sample t-test:

$$\frac{|\bar{x}_c - \bar{x}_t|}{S\sqrt{\frac{1}{n_c} + \frac{1}{n_t}}} > t(1-\alpha)(n_c + n_t - 2)$$

$$\text{where } S = \frac{S(x_c - \bar{x}_c)^2 + S(x_t - \bar{x}_t)^2}{n_c + n_t - 2}$$

IV. EXFERIMENTAL RESULTS

EXPERIMENTAL RESULTS

I. BIOLOGICAL CHARACTERIZATION OF REAGENTS

1. SE Antigens

All lyophilized SE antigens contained similar amounts of protein, calculated as mg protein per mg dry weight (Table I). Infection with H1 virus did not consistently alter the total amount of soluble cellular protein.

Viral SE antigens H-R/SE and H-F/SE contained only small amounts of residual infective virus (Table II). Comparison of the infectivity titers of H-R/SE and H-F/SE with those of the original cell extracts from which they were derived showed that infective virus was almost completely removed by the two cycles of high-speed centrifugation used in the preparative procedure.

Preliminary immunodiffusion experiments, outlined in Appendix V, in which various dilutions of H-R/SE and H-F/SE were tested against selected antisera, showed that the maximum number of precipitin lines was produced when the SE antigen concentration was 8 mg protein per ml. Therefore, all SE antigens were standardized to contain this amount of protein.

TABLE I

PROTEIN CONTENT OF LYOPHILIZED SE ANTIGENS RECONSTITUTED IN 2 ML H_2O

SE Antigen*	Dry weight mg	Protein** mg/ml	Total protein / Dry Weight
a) RK13-derived			
H-R/SE	88.3	17	0.39
R/SE	80.0	14	0.35
b) F-derived			
H-F/SE	130	21.5	0.33
F/SE	140	25	0.36

* Soluble antigens of cell extracts: H-R/SE and H-F/SE of H1-infected cells, and R/SE and F/SE of uninfected cells

** Determined by the Lowry method

TABLE II

REMOVAL OF INFECTIVE H1 VIRIONS FROM SE ANTIGENS BY PREPARATIVE ULTRACENTRIFUGATION

Preparation	Volume (ml)	Infectivity* (TCD_{50}/ml)
H1-infected RK13 cell extract	10	$10^{8.7}$
H-R/SE	2	$10^{2.8}$
H1-infected F cell extract	10	$10^{7.8}$
H-F/SE	2	$10^{2.0}$

* Assayed by micro-titer plate method in RK13 cells

2. Virion Antigens and Control Antigens

a) Concentration of Crude ($-C_2$) Antigens

Differential centrifugation was found to be an effective method for the concentration and preliminary purification of crude virion antigens H-RC_2 and H-FC_2. Comparison of the results of protein determinations, infectivity assays, and particle counts by electron microscopy performed on samples of antigens at different stages of the concentration procedure (Table III) showed that concentration of virions, with about 50% recovery, was achieved at the same time as significant amounts of contaminating protein were removed.

The protein content of control host antigens from uninfected RK13 or F cells is also shown in Table III. Since control antigens R-C_2 and F-C_2 contained similar amounts of protein as the corresponding crude viral antigens H-RC_2 and H-FC_2, these control antigens were considered suitable for use in immunodiffusion tests.

b) Purification of Virion Antigens H-R/V and H-F/V

Centrifugation through sucrose density gradients resulted in considerable purification of virion antigens. Electron microscopy of virion antigens before and after centrifugation (Plate 1, figures a and b) demonstrated the presence of both enveloped and naked H1 virions of typical HSV morphology, which were easily distinguished by their capsid structure from the PSL spheres used for the counting technique. Membranous and amorphous debris was more prominent in crude than in purified virion antigen preparations.

TABLE III

CONCENTRATION OF VIRION AND CONTROL ANTIGENS BY DIFFERENTIAL CENTRIFUGATION: MEASURED BY PROTEIN DETERMINATIONS, INFECTIVITY TITRATIONS, AND VIRUS PARTICLE COUNTS

Stage	Volume (ml)	Protein (mg/ml)*	Infectivity (TCD_{50}/ml)**	Virus Particle Count (per ml)***
a) RK13-grown: Viral				
1. Original medium	720	-#	$10^{8.0}$	1.3×10^{9}
2. H-RC_1 (first concentrate)	12	1200	$10^{9.0}$	7.9×10^{10}
3. H-RC_2 (crude virion antigen)	2.4	1100	$10^{9.8}$	3.4×10^{11}
b) RK13-grown: Control				
1. Original medium	720	-	-	-
2. R-C_1 (first concentrate)	12	1250	-	-
3. R-C_2 (control antigen)	2.5	1200	-	-
c) F-grown: Viral				
1. Original medium	800	-	$10^{6.3}$	1.0×10^{9}
2. H-FC_1 (first concentrate)	20	1600	$10^{7.5}$	2.6×10^{10}
3. H-FC_2 (crude virion antigen)	2.4	1400	$10^{9.0}$	7.4×10^{11}

continued....

TABLE III (Continued)

Stage	Volume (ml)	Protein (mg/ml)*	Infectivity (TCD_{50}/ml)**	Virus Particle Count (per ml)***
d) F-grown: Control				
1. Original medium	800	-#	-	-
2. F-C_1 (first concentrate)	12.5	1960	-	-
3. F-C_2 (control antigen)	2.4	1400	-	-

* Estimated by the Lowry method

** Assayed in RK13 cells in micro-titer plates

***Determined by direct ratio to standard PSL by electron microscopy

Not done

PLATE I

MORPHOLOGY OF H1 (HSV) VIRIONS: H-RC$_2$ (CRUDE) AND H-R/V (PURIFIED) RK13-GROWN VIRUS SUSPENSIONS

Legend

PSL - Polystyrene latex spheres

Figure a) H-RC$_2$ crude virion suspension. Virus particles are easily distinguished by their capsid structure from PSL spheres.

Figure b) H-R/V virions after purification by sucrose density gradient centrifugation.


β
α
200nm

Comparison of the protein content and virus particle counts of crude and purified virion antigens (Table IV) showed that over half the protein was removed from crude antigens by one cycle of sucrose density gradient centrifugation, with recovery of 60-100% of the virion particles.

Hl virion antigens H-R/V and H-F/V were not regarded as being highly purified by this relatively simple procedure. The protein: particle ratio of 25 µg. protein per 10^{10} virions, shown for H-R/V in Table IV, however, compares favorably with recent results reported by Robinson and Watson (1971). They achieved protein: particle ratios of 4.2 µg. protein per 10^{10} virus particles in suspensions of virus which they considered to be very highly purified after exhaustive treatment with fluorocarbons and enzymes, density gradient centrifugation, and calcium phosphate column separation techniques. These procedures resulted in the recovery of less than 3% of the original virus particles, and the almost complete loss of viral envelopes.

It was considered that, for the purposes of immunological analysis by immunodiffusion techniques, sufficient purification of virion antigens H-R/V and H-F/V had been achieved by one cycle of sucrose density gradient after concentration and preliminary purification of virions by differential centrifugation. Only extracellular virus was used as the source of virion antigens, providing a much "cleaner" starting preparation than the infected cell lysates used by Robinson and Watson as their starting material.

TABLE IV

PURIFICATION OF H1 VIRION ANTIGENS BY SUCROSE DENSITY GRADIENT CENTRIFUGATION: COMPARISON OF PROTEIN DETERMINATIONS AND VIRUS PARTICLE COUNTS OF CRUDE AND PURIFIED VIRION SUSPENSIONS.

Antigen	Volume (ml)	Particle Count (per ml)*	Protein (ug/ml)**	$\frac{\text{Protein (ug)}}{\text{Particles x10}}$ 10
a) RK13 grown				
H-RC_2 (crude)	2.4	2.1×10^{11}	1200	60
H-R/V (purified)	2.5	2.8×10^{11}	700	25
b) F cell-grown				
H-FC_2 (crude)	2.5	3.1×10^{10}	900	300
H-F/V (purified)	2.5	1.8×10^{10}	250	138

* Determined by direct ratio to standard PSL by electron microscopy

** Estimated by the Lowry method

3. Analytical Antisera

a) Neutralization Titers against H1-R Virus Suspensions

The antisera selected for the characterization and comparative analysis of viral antigens are listed, together with their immunizing antigens and virus neutralization titers, in Table V. The titers of neutralizing antibody in hyperimmune and convalescent antisera indicated a satisfactory immune response, and hence these sera were considered suitable for the analysis of viral and host antigens by immunodiffusion.

No neutralizing antibody was detected when RaF, anti-host serum produced against host antigens of the heterologous F cell system, was tested against stock RK13 cell-grown virus H1-R. Anti-host serum RaR, on the other hand, produced against host antigens of homologous RK13 cells, apparently contained low amounts of antibody. This finding was not unexpected, in view of the possibility that RaR may have reacted with antigenic viral receptor sites on susceptible RK13 cells, and thus interfered with viral adsorption.

b) Immunodiffusion Characteristics

Preliminary experiments, described in Appendix V, were performed to test the effects of inactivation and dilution of some antisera on their immunodiffusion reactions. The results showed that undiluted sera, either native or inactivated at 56°C for thirty minutes, detected the maximum number of precipitin lines. Therefore, all antisera were used undiluted and were not inactivated for analytical immunodiffusion tests.

TABLE V

CHARACTERISTICS OF THE ANTISERA USED FOR ANALYSIS OF H1 VIRAL AND HOST CELL ANTIGENS

Serum		Immunizing Viral Antigen	Neutralization * Titer	Immuno-diffusion **
a) Hyperimmune rabbit sera				
1. To antigens derived from RK13 cells				
RaR	anti-host	-	20	0
RaHR-V	anti-virion	H-R/V	1920	1-2
RaHR-S	anti-SE	H-R/SE	640	8-10
2. To antigens derived from F cells				
RaF	anti-host	-	<10	1-2
RaHF-V	anti-virion	H-F/V	160	2-3
RaHF-S	anti-SE	H-F/SE	1280	7-8
b) Convalescent Sera				
RaHco	convalescent rabbit serum		800	-
HuH	human serum, from the same person from whom H1 was isolated		160	-
HuGG	pooled human immune globulin		160	-
c) Normal Serum				
NRS	normal rabbit serum		<10	-

* determined for 100 TCD_{50} H1-R in RK13 cells in micro-titer plates; reciprocal of 50% endpoint dilution, calculated by Reed-Muench method.

** number of precipitin lines produced in immunodiffusion reactions between antiserum and its immunizing antigen.

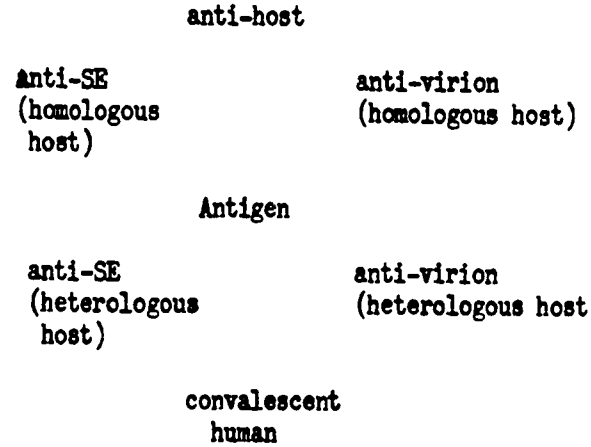
A standard reaction pattern was used for the characterization of reagents by immunodiffusion. Six different antisera were placed in the peripheral wells, surrounding antigen in the centre well. The pattern is shown diagrammatically in Figure 2; the position of the antiviral sera was varied according to the host origin of the antigen in the centre well, so that antisera homologous to the host were always placed in the three top wells.

Immunodiffusion reactions, set up according to this standard pattern for the initial characterization of seven analytical antisera are shown in Plate 2. Reactions between these antisera and crude control host antigens R-ex (RK13 cell host) and F-ex (F cell host) are presented in figures a and c, respectively, and are compared with the reactions between the same antisera and crude viral antigens H-R-ex and H-F-ex, presented in figures b and d. The results show that:

i) None of the antisera reacted with host antigens of R-ex (figure a). This finding agrees with the results of Watson et al (1966), and justifies the initial decision to use RK13 cells (grown in medium supplemented with normal rabbit serum instead of fetal calf serum) for the propagation of viral antigens,, so that normal host cell antigens, which would not elicit antibody formation during immunization of rabbits, could thus be excluded from the test reaction system.

FIGURE 2

STANDARD REACTION PATTERN FOR IMMUNODIFFUSION CHARACTERIZATION OF ANALYTICAL ANTISERA AND VIRAL AND HOST ANTIGENS

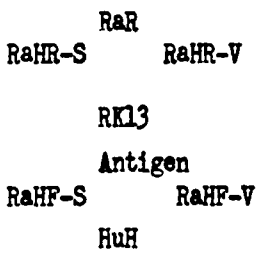


Antisera to:

Homologous Host - Hyperimmune serum prepared against viral antigens derived from the same host cell as the antigens being tested.

Heterologous Host - Hyperimmune serum prepared against viral antigens derived from the alternate host cell.

i.e. for RK 13 - derived antigens:



and for F-derived antigens:

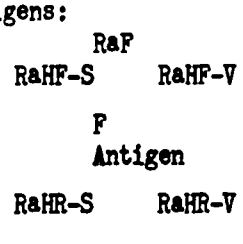


PLATE 2

CHARACTERIZATION OF ANALYTICAL ANTISERA BY IMMUNODIFFUSION AGAINST ANTIGENS OF H1-INFECTED OR UNINFECTED RK13 OR F CELLS

Legend

Antigens (centre well):

R-ex crude antigens of uninfected RK13 cells
H-R-ex crude antigens of H1-infected RK13 cells

F-ex crude antigens of uninfected F cells
H-F-ex crude antigens of H1-infected F cells

Antisera (peripheral wells):

a) Rabbit antisera:
RaR to R-C_2: RK13 host control antigen
RaHR-V to H-R/V: purified H1 virions grown in RK13 cells
RaHR-S to SE antigens of H1-infected RK13 cells

RaF to F-C_2: F cell host control antigens
RaHF-V to H-F/V: purified H1 virions grown in F cells
RaHF-S to SE antigens of H1-infected F cells

b) Human serum
HuH convalescent serum

Figure a) Reactions of analytical antisera with R-ex.
None of the analytical antisera react with host antigens of R-ex.

Figure b) Reactions of analytical antisera with H-R-ex.
There is no reaction between RaR and H-R-ex antigens. Homologous antiserum RaHR-S reacts with at least seven antigenic components; and heterologous antiviral serum RaHF-S also reacts with at least two of these (arrowed reactions of identity). Homologous RaHR-V and heterologous RaHF-V anti-virion sera both react with at least two viral antigens, and HuH reacts with at least one of these (reaction of identity double-arrowed), as well as with three other viral antigens.

Figure c) Reactions of analytical antisera with F-ex. Homologous antisera RaF, RaHF-V, and RaHF-S all react with normal F host antigens. RaHF-S detects at least three normal host antigens, and RaF and RaHF-V each detect at least one. Heterologous antisera RaHR-V and RaHR-S, and HuH do not react with normal F antigens.

Figure d) Reactions of analytical antisera with H-F-ex. Anti-host serum RaF reacts with at least one host antigen of H-F-ex, and homologous anti-SE serum RaHF-S reacts with the same host antigen (arrowed reaction of identity) as well as with at least four other antigens. Antibody to three of these antigens is also present in RaHR-S, as well as in HuH, RaHR-V, and RaHF-V (reactions of identity).

Ra
R
Rc
HR-S
Ra
HR-V
R-ex
Ra
HF-S
Ra
HF-V
Hu
H
a
Rc
R
Ra
HR-S
Ra
HR-V
H-R
ex
Ra
HF-S
Ra
HF-V
Hu
H
b
Ra
F
Ra
HF-S
Ra
HF-V
F-ex
Ra
HR-S
Ra
HR-V
Hu
H
c
Ra
F
Ra
HF-S
Ra
HF-V
H-F
ex
Ra
HR-S
Ra
HR-V
Hu
H
d

ii) Anti-host serum RaR, as expected, did not react with host antigens of H-R-ex (figure b). Antibodies reacting with several antigens were, however, present in homologous antiviral sera RaHR-V and RaHR-S, produced against antigens of H1-infected RK13 cells. Some of these antibodies were characterized as antiviral by their reactions of identity, produced to three antigens, with corresponding heterologous antiviral sera RaHF-V and RaHF-S; and antibodies reacting with at least two of the same antigens was also present in human serum HuH. Antibodies in homologous anti-SE serum RaHR-S detected at least eight antigenic components; in contrast, homologous anti-virion serum RaHR-V reacted with only three viral antigens.

iii) Anti-host serum RaF, against normal F cell antigens, and homologous antiviral sera RaHF-V and RaHF-S all contained antibody to normal host antigens of F-ex (figure c). No antibody to normal F antigens was apparent in heterologous antiviral sera RaHR-V or RaHR-S, or in human serum HuH.

iv) Antibody reacting with the same F cell host antigen was apparent in the three homologous antisera, shown by the reaction of identity (figure d) when they were tested against F-ex viral antigens. However, as shown by reactions of identity with heterologous antiviral sera RaHR-V and RaHR-S, and with human serum HuH, antibodies to at least two viral antigens were also present in antiviral sera RaHF-V and RaHF-S.

2. CHARACTERIZATION OF ANTIGENS BY IMMUNODIFFUSION

1. SE Antigens

a) Characterization of the Reactions between SE Antigens and Analytical Antisera

Immunodiffusion characteristics of H-R/SE and H-F/SE (SE antigens of Hl-infected RK13 and F cells) tested against analytical antisera are shown in Plate 3 (figures a and b).

H-R/SE (figure a) did not react with anti-host serum RaR. At least eight antigenic components, however, reacted with homologous anti-SE serum RaHR-S, and six with homologous anti-virion serum RaHR-V; some of these may have been host or modified-host antigens recognizable only by homologous antisera, but at least three were viral-specific, shown by their reactions of identity with heterologous sera RaHF-S and RaHF-V; and at least two of these were shown by their reactions with human convalescent serum HuH to be antigens which are recognized in human infection.

Similar reactions were produced between antigens of H-F/SE and the same analytical antisera (figure b); however, in this case host antigens of H-F/SE were recognized by their reactions with anti-host serum RaF. One, and possibly two of these host antigens also reacted with homologous antiviral sera RaHF-S and RaHF-V. At least four other antigenic components were detected by anti-SE serum RaHF-S, and two by anti-virion serum RaHF-V; as with antigens of H-R/SE, at least two of these were shown to be of viral origin by the reactions of identity between heterologous antiviral sera RaHR-S and RaHR-V and human serum HuH.

PLATE 3

IMMUNODIFFUSION REACTIONS OF VIRAL SE ANTIGENS H-R/SE AND H-F/SE

Legend

Antigens:

H-R/SE	SE antigens of H1-infected RK13 cells
H-F/SE	SE antigens of H1-infected F cells

Antisera:

a) Rabbit hyperimmune sera:

RaR	to R-C_2: RK13 cell host control antigens
RaHR-V	to H-R/V: purified H1 virions grown in RK13 cells
RaHR-S	to H-R/SE
RaF	to F-C_2: F cell host control antigens
RaHF-V	to H-F/V: purified H1 virions grown in F cells
RaHF-S	to H-F/SE

b) human:

Huh	convalescent serum

Figure a) Reactions of H-R/SE in cellulose acetate. No host antigens are detected by RaF. At least eight antigens react with homologous serum RaHR-S; at least three of these are viral antigens, shown by reactions of identity (arrowed) with RaHF-S, and two also react with HuH. One of the same antigens reacts with anti-virion sera RaHF-V and RaHR-V, and two other viral antigens also react with these sera (reactions of identity arrowed). At least three other antigens are detected only by homologous anti-virion serum RaHR-V.

Figure b) Reactions of H-F/SE in cellulose acetate. The presence of a normal host antigen in H-F/SE is shown by its reaction with RaF. Several antigens react with homologous serum RaHF-S; the viral origin of two of these is shown by the reactions of identity (arrowed) with heterologous anti-SE serum RaHR-S.

Figure c) Reactions of H-R/SE in 1% agarose.
The presence of multiple antigens in H-R/SE is shown by the formation of several precipitin lines (compare with figure a).

Figure d) Reactions of H-F/SE in 1% agarose.
Several antigens of H-F/SE react with analytical antisera; the precipitin lines are not easily resolved (compare with figure b).

Ra
R
Ra
HR-V
H-R
SE
Ra
HF-V
Hu
H
a
Ra
F
Ra
HF-V
H-F
SE
Ra
HR-V
Hu
H
b
Ra
R
Ra
HR-V
H-R
SE
Ra
HF-V
Hu
H
c
Ra
F
Ra
HF-V
H-F
SE
Ra
HR-V
Hu
H
d

a
b
Ra
HR-V
Ra
HF-S
Ra
HF-V
Ra
HR-S
d

b) Selection of Support Medium

The suitability of cellulose acetate for immunodiffusion studies of these SE antigens was tested by comparison with 1% agarose, used in previous studies of HSV antigens (Watson et al, 1966). The results of parallel immunodiffusion reactions in both media are shown in Plate 3. The reactions of H-R/SE are compared in figures a and c, and those of H-F/SE in figures b and d. Precipitin lines were more sharply-defined and easily-resolved in cellulose acetate (figures a and b) than in agarose (figures c and d). Cellulose acetate was also more sensitive; in the reaction between H-R/SE and RaHR-S, for example, eight antigenic components were detected in cellulose acetate (figure a) but only six of these were detected in agarose (figure c). Identification of two components of H-F/SE as viral antigens was achieved in cellulose acetate, in which linkages formed between antisera RaHF-S and RaHR-S (figure b); but this was not possible using agarose (figure d).

c) Further Characterization of SE Antigens by Immunodiffusion Reactions with Selected Antisera

The immunodiffusion reactions of SE antigens were further characterized as shown in Plate 4.

The immunodiffusion reactions of F/SE, (SE antigens of uninfected F cells), are shown in figure a, and those of R/SE, (SE antigens of uninfected RK13 cells), in figure b. At least one host antigen of F/SE reacted with homologous antisera RaF, RaHF-V, and RaHF-S, but not with heterologous antisera RaHR-V, RaHR-S, or with human serum HuH (figure a). R/SE, on the other hand (figure b) contained no host antigens reacting with any of the analytical

PLATE 4

FURTHER CHARACTERIZATION OF SE ANTIGENS BY IMMUNODIFFUSION REACTIONS AGAINST SELECTED ANTISERA

Legend

Antigens:

R/SE	SE antigens of uninfected RK13 cells
F/SE	SE antigens of uninfected F cells
H-R/SE	SE antigens of H1-infected RK13 cells
H-F/SE	SE antigens of H1-infected F cells

Antisera:

a) Rabbit antisera:

RaR	to R-C2: RK13 cell host control antigens
RaHR-V	to H-R/V: purified H1 virions grown in RK13 cells
RaHR-S	to H-R/SE
RaF	to F-C_2: F cell host control antigens
RaHF-V	to H-F/V: purified H1 virions grown in F cells
RaHF-S	to H-F/SE
NRS	normal rabbit serum

b) human sera:

HuH	convalescent serum
HuGG	pooled human immune globulin

Figure a) Reactions of F/SE.
At least one host antigen of R/SE reacts with homologous antisera RaF, RaHF-V, and RaHF-S. No reaction is produced between F/SE and heterologous antisera RaHR-V or RaHR-S, or human serum HuH.

Figure b) Reactions of R/SE.
R/SE host antigens do not react with any of the antisera.

Figure c) Reactions of H-F/SE.
No reaction is produced between H-F/SE and NRS. At least four antigens react with homologous antiserum RaHF-S, and at least six viral antigens with heterologous anti-SE serum RaHR-S. At least three viral antigens are defined by their reactions with heterologous anti-virion serum RaHR-V.

Figure d) Reactions of H-R/SE.
No reaction is produced between H-R/SE and NRS. At least six antigens react with homologous serum RaHR-S, and at least four with homologous anti-virion serum RaHR-V. The reactions of at least two antigens with heterologous antiviral sera RaHF-S and RaHF-V show their viral origin.

Figure e) Reactions of H-F/SE.
At least four antigens of H-F/SE react with homologous antiserum RaHF-S. (cf figure c), and at least two with homologous anti-virion serum RaHF-V. One, at least, of these antigens is shown to be of viral origin by its reaction of identity (arrowed) between RaHF-V, HuH, and HuGG. The presence of at least one (and possibly two) other major viral antigens is shown by its reaction of identity with both HuH and HuGG.

Figure f) Reactions of H-R/SE.
At least four precipitin lines are produced between H-R/SE and homologous antiserum RaHR-S, and at least two between H-R/SE and RaHR-V. The viral origin of one of these antigens is proven by its reaction with HuH and HuGG (reactions of identity are arrowed).

Ra F
Ra HF-S
Ra HF-V
F SE
Ra HR-S
Ra HR-V
Hu H
a
Ra R
Ra HR-S
Ra HR-V
R SE
Ra HF-S
Ra HF-V
Hu H
b
Ra HR-S
Ra HR-V
NRS
H-F SE
Ra HF-S
Ra HR-V
Ra HF-V
c
Ra HR-S
Ra HR-V
NRS
HR SE
Ra HF-S
Ra HR-V
Ra HF-V
d
Ra HF-S
Hu H
Hu GG
H-F SE
Hu GG
Hu H
Ra HF-V
e
Ra HR-S
Hu H
Hu GG
HR SE
Hu GG
Hu H
Ra HR-V
f

antisera; this was expected, on the basis of previous results (Plate 2).

H-F/SE and H-R/SE were compared by setting up parallel reactions of each with identical arrangements of antisera in the peripheral wells (figures c and d; e and f). The results showed that:

i) Neither H-F/SE (figure c) or H-R/SE (figure d) reacted with normal rabbit serum NRS. Specific antibody was required for the formation of precipitin lines. Some SE preparations, particularly those produced early in the work, produced the broad hazy zones around the antigen well which are visible in figures c and d. Such zones, although they did not appear to influence the formation of precipitin lines, made the counting of the lines more difficult. SE antigens produced later in the work did not produce the same hazy zones. It was thought that they were due to the presence of lipid contamination, which progressively decreased as purification techniques improved.

ii) The maximum number of antigens was detected in both H-F/SE and H-R/SE by antiviral SE serum RaHR-S. For example, H-F/SE contained at least six viral antigens detected by RaHR-S, but only four (some of which may have been host antigens) were detected by homologous serum RaHF-S (figure c). Similarly, seven antigens of H-R/SE were detected by RaHR-S but only four by heterologous serum RaHF-S (figure d). This result may have represented a fundamental

difference in the properties of H-R/SE and H-F/SE; it was more likely due to differences in the potency of the respective immunizing antigens, and/or differences in the responses of individual rabbits.

iii) Four antigenic components of H-F/SE reacted with RaHF-S, and at least three with homologous anti-virion serum RaHF-V (figure e). No conclusions regarding the origin of these antigens could be drawn from these results; it was possible that some, at least, were of host origin, since these antisera also reacted with antigens of control F/SE (figure a).

iv) The presence of at least two major viral antigens in H-F/SE and H-R/SE was shown by their reactions (figures e and f) with human serum HuH and pooled human immune globulin HuGG. These two antigens must be common to all HSV strains, since HuGG contained pooled human antibody produced as a result of widespread natural infection by wild strains.

On the basis of these results, it was concluded that SE antigens H-F/SE and H-R/SE each contained several antigenic components of viral origin. All antigens detected in H-R/SE were of viral or viral-modified host origin, since a) no antigens were detected by analytical antisera in R/SE control antigens (of uninfected RK13 cells), and b) antiserum RaR to control R-C_2 host antigens did not react with any antigens of H-R/SE. Some antigens of H-F/SE detected by homologous antisera RaHF-S and RaHF-V, however, may have been of host origin, since these antisera reacted with host antigens

of F/SE, and since anti-host serum RaF reacted with some antigens of H-F/SE. The use of antisera directed against viral antigens produced in the two hosts, RK13 and F cells, and of human antisera HuH and HuGG, permitted the identification of at least two major viral antigens, recognized by human convalescent sera, and therefore common to several strains of HSV, as well as of at least five other viral antigens, identified by their reactions with heterologous antisera.

d) Identification of Viral Antigens of H-R/SE and H-F/SE

The viral origin of two antigens detected in H-R/SE and H-F/SE by both homologous and heterologous antiviral sera (Plates 3 and 4) was confirmed by the results shown in Plate 5. The two identical antigens of H-R/SE and H-F/SE reacted with both homologous and heterologous anti-SE sera RaHR-S and RaHF-S (figures a and b), and two virion antigens reacted with anti-virion antisera RaHR-V and RaHF-V (figure a). In addition, some antigenic components of each viral SE antigen were detected only by homologous anti-SE serum; and some antigens were present which gave reactions of partial identity, shown by splitting of the precipitin lines formed by these antigens of H-F/SE when they merged with the precipitin lines formed by some components of H-R/SE. Such reactions of incomplete identity may result from a) aggregation of antigenic components, or b) host modification of viral antigens.

PLATE 5

IDENTIFICATION OF VIRAL ANTIGENS IN H-R/SE AND H-F/SE
SE ANTIGENS BY IMMUNODIFFUSION REACTIONS

Legend

Antigens:

H-R/SE	SE antigens of H1-infected RK13 cells
H-F/SE	SE antigens of H1-infected F cells

Rabbit antisera:

RaHR-V	to H-R/V: purified H1 virions grown in RK13 cells
RaHR-S	to H-R/SE
RaHF-V	to H-F/V: purified virions grown in F cells
RaHF-S	to H-F/SE

Figure a) Reactions of H-R/SE and H-F/SE.
Two viral antigens of H-R/SE and H-F/SE are identified by reactions of identity (arrowed) between both H-R/SE and H-F/SE with anti-SE sera RaHR-S and RaHF-S. Two viral antigens (which may be the same) are similarly identified by the reactions of identity (double-arrowed) between H-R/SE and H-F/SE and anti-virion sera RaHR-V and RaHF-V. A reaction of partial identity between at least one antigen of H-F/SE and H-R/SE is shown by splitting of the precipitin line.

Figure b) Reactions of H-F/SE and H-R/SE.
The presence of the two viral antigens common to H-R/SE and H-F/SE is confirmed in this reaction (reactions of identity are arrowed). At least two antigens give reactions of partial identity between H-F/SE and H-R/SE, shown by splitting of the precipitin lines.

H-F
SE
Ra
HF-S
Ra
HF-V
H-R
SE
Ra
HR-S
Ra
HR-V
H-F
SE
a
H-R
SE
Ra
HF-S
Ra
HF-V
H-F
SE
Ra
HR-S
Ra
HR-V
H-R
SE
b

2. Virion and Control Antigens

a) Characterization of the Immunodiffusion Reactions of Virion and Control Antigens

Plate 6 characterizes the immunodiffusion reactions of RK13 cell-derived R-C_2 host control antigens and H-R/V virion antigens (figures a and b), and of F cell-derived F-C_2 host control antigens and H-F/V purified virion antigens (figures c and d).

Normal host antigens of R-C_2 did not react with any of the analytical antisera (figure a), thus confirming previous observations (Plates 2 and 4) that rabbits immunized with RK13 cell-derived antigens produced no antibody against normal cell constituents. H-R/V, on the other hand, (figure b) contained at least one virion antigen which reacted with homologous and heterologous antiviral sera, and with human serum HuH, as well as with anti-host serum RaR. This reaction of H-R/V with RaR occurred with several batches of H-R/V virion antigens. It could be due to a)inefficient purification of virion antigens, resulting in the selective concentration of host cell antigens, as well as of virions, b) selective incorporation into infectious virions of a normal cell antigen which was present but below detectable levels in control antigen preparations, or c) incorporation into Hl virions of a viral-modified host component which was not reactive in its unaltered state with the analytical antisera, thus resulting in a new antigen with dual host-virus specificity.

Host antigens of F-C_2 control antigens (figure c) reacted

PLATE 6

IMMUNODIFFUSION REACTIONS OF VIRION AND CONTROL ANTIGENS

Legend

Antigens:

$R\text{-}C_2$	RK13 cell host control antigens
H-R/V	purified H1 virions grown in RK13 cells
$F\text{-}C_2$	F cell host control antigens
H-F/V	purified H1 virions grown in F cells

Antisera:

a) Rabbit hyperimmune sera:

RaR	to $R\text{-}C_2$
RaHR-V	to H-R/V
RaHR-S	to H-R/SE: SE antigens of H1-infected RK13 cells
RaF	to $F\text{-}C_2$
RaHF-V	to H-F/V
RaHF-S	to H-F/SE: SE antigens of H1-infected F cells

b) Human serum

HuH	convalescent serum

Figure a) Reactions of $R\text{-}C_2$ in cellulose acetate.
No host antigens of $R\text{-}C_2$ react with any of the antisera.

Figure b) Reactions of H-R/V in cellulose acetate.
One component of H-R/V reacts (reaction of identity) with all antisera. A second antigen is detected only by homologous anti-virion serum RaHR-V.

Figure c) Reactions of $F\text{-}C_2$ in cellulose acetate.
At least two host antigens are detected by both RaF and RaHF-S with reactions of identity. One host antigen reacts with homologous anti-virion serum RaHF-V. No reaction is produced between $F\text{-}C_2$ and heterologous sera RaHR-S or RaHR-V, or human serum HuH.

Figure d) Reactions of H-F/V in cellulose acetate.
One viral antigen is identified by its reactions with heterologous antiviral sera RaHR-V and RaHR-S, and with HuH (reactions of identity arrowed). One host antigen reacts with RaF and RaHF-S. Two antigens are detected by RaHF-V; they may be of either viral or host origin.

Figure e) Reactions of H-R/V in 1% agarose.
At least one antigen of H-R/V reacts with all antiviral sera; homologous antiviral sera RaHR-S and RaHR-V each detect a second antigen. No reaction is produced with anti-host serum RaR. (compare with figure b.)

Figure f) Reactions of H-F/V in 1% agarose.
Only one antigen of H-F/V is detected, by its reaction with homologous anti-virion serum RaHF-V. (compare with figure d.)

Ra R
Ra HR-S
Ra HR-V
R-C2
Ra HF-S
Ra HF-V
Hu H
a
Ra R
Ra HR-S
Ra HR-V
H-R V
Ra HF-S
Ra HF-V
Hu H
b
Ra F
Ra HF-S
Ra HF-V
F-C2
Ra HR-S
Ra HR-V
Hu H
c
Ra F
Ra HF-S
Ra HF-V
H-F V
Ra HR-S
Ra HR-V
Hu H
d
Ra R
Ra HR-S
Ra HR-V
H-R V
Ra HF-S
Ra HF-V
Hu H
e
Ra F
Ra HF-S
Ra HF-V
H-F V
Ra HR-S
Ra HR-V
Hu H
f

with anti-host serum RaF, and with homologous antiviral sera RaHF-V and RaHF-S. No antigens of F-C_2 reacted with heterologous antiviral sera RaHR-V or RaHR-S, or with human serum HuH.

Virion antigens H-F/V (F cell-derived) also contained normal F cell antigens (figure d). One such unmodified host antigen, identified by its reaction with RaF, also reacted with homologous anti-SE serum RaHF-S. At least one viral-specific antigenic component was identified by its reaction with heterologous antiviral sera RaHR-S and RaHR-V, and with HuH. Two antigenic components reacted with homologous anti-virion serum RaHF-V; in view of the fact that RaHF-V also reacted with host antigens of F-C_2 (figure c) they could not be identified as viral antigens on the basis of this reaction.

b) Selection of Support Medium

The precipitin lines formed when Hl virion antigens H-R/V and H-F/V reacted with antiviral sera in cellulose acetate were rather fuzzy and faint (figures b and d). For this reason, and because of reports that HSV virions did not diffuse in standard agar gel immunodiffusion (Robinson and Watson, 1971), cellulose acetate and 1% agarose immunodiffusion reactions were set up in parallel to compare the suitability of these two media for immunodiffusion studies of Hl virion antigens.

H-R/V contained one component which reacted with all the antiviral sera tested in 1% agarose (figure e). No reaction was visible in agarose between H-R/V antigens and anti-host serum RaR, although this reaction was very clear in cellulose acetate (figure b).

Only one antigenic component of H-F/V was detected in 1% agarose (figure f), by its reaction with homologous anti-virion serum RaHF-V, although in cellulose acetate (figure d) at least two antigens of H-F/V reacted with RaHF-V, and at least one other with heterolcgous antiviral sera RaHR-V and RaHR-S, and with human serum HuH.

These results, similar to earlier results obtained with SE antigens (Plate 3), show that cellulose acetate was more sensitive than agarose for the detection of virion antigens.

The pore size of 1% agarose has been calculated to be 60-100 nm in diameter (Ackers and Steere, 1962). This is too small to allow the free diffusion of particles the size of HSV virions, of diameters 100 (naked) to 180 (enveloped) nm. It has been determined (Cliver, 1968) that diffusion of virus particles through cellulose membranes requires that the pore size of the membrane be from two to three times the diameter of the virus particle. The cellulose acetate membranes used in this study were designed for electrophoresis, and according to manufacturer's specifications, consisted of pores ranging in diameter from 50-300 nm. When H-R/V virions were tested against analytical sera by immunodiffusion in a sample of 'Celotate' cellulose acetate of known standard pore size 200 nm (Millipore Filter Co.) no precipitin lines were produced. It is assumed, therefore, that diffusion of virions through the larger pores of the membrane, that is, those from 200-300 nm diameter, is responsible for the precipitin reactions produced between virion antigens and analytical antisera in immunodiffusion in cellulose acetate.

c) Immunodiffusion Reactions of H-R/V after Treatment with SDS, DOC, Ether, and Trypsin.

The effects of treating H-R/V (purified RK13 cell-grown H1 virions) with disrupting agents SDS, DOC, ether, and trypsin were assessed by immunodiffusion reactions in which the reactions of intact and treated virions were compared, using anti-virion antisera RaHR-V and RaHF-V. The results, presented in Plate 7, showed that a 'new' structural antigen was apparent after treatment of H-R/V with DOC. This antigen, which was not detected in intact H-R/V virions, or in H-R/V treated with SDS, ether, or trypsin, reacted with both anti-virion sera RaHR-V and RaHF-V. Other experiments showed that it did not react with anti-host serum RaR, or with NRS control serum.

DOC was thus concluded to be the most suitable of the disrupting agents tested, resulting in the release of one 'new' structural antigen from H-R/V, and DOC-treated H-R/V was therefore used in the comparative analyses of virion and soluble antigens described in Section IV, designed to permit the identification of soluble viral structural antigens.

Treatment with SDS or DOC resulted in solubilization of H-R/V as seen by clarification of the virus suspension. H-R/V suspensions treated with ether or trypsin remained cloudy, and these latter suspensions were examined by electron microscopy. No consistent alteration of virion morphology could be identified as a result of either of these treatments; intact enveloped virions

indistinguishable from those of control preparations predominated in treated suspensions, even though the results of infectivity assays showed that infectivity was markedly reduced by trypsin treatment, and totally abolished by ether. Wildy et al (1960) also treated HSV with ether; the only consistent difference in virion morphology they detected as a result was an increase in the numbers of viral envelopes enclosing more than one nucleocapsid.

PLATE 7

IMMUNODIFFUSION REACTIONS OF DISRUPTED VIRIONS

Legend

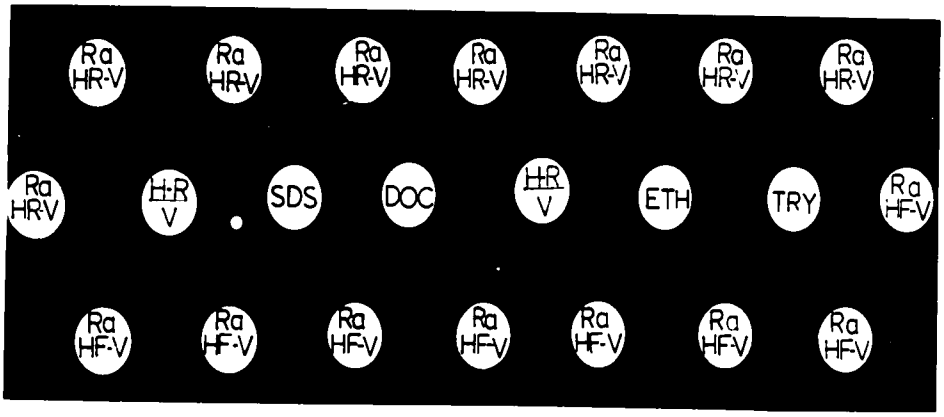
Antigens:

H-R/V	purified H1 virions grown in RK13 cells
SDS	SDS-treated H-R/V
DOC	DOC-treated H-R/V
ETH	Ether-treated H-R/V
TRY	Trypsin-treated H-R/V

Rabbit antisera:

RaHR-V	to H-R/V: purified H1 virions grown in RK13 cells
RaHF-V	to H-F/V: purified H1 virions grown in F cells

Reactions of treated and control H-R/V.
Two antigens of H-R/V are demonstrated by their reactions with RaHR-V, and one of these also reacts with RaHF-V. No antigens are detectable in SDS, ETH, or TRY, but DOC contains a 'new' antigenic component which reacts with both anti-virion sera.


Ra HR-V
Ra HR-V
Ra HR-V
Ra HR-V
Ra HR-V
Ra HR-V
Ra HR-V
Ra HR-V
H-R V
SDS
DOC
H-R V
ETH
TRY
Ra HF-V
Ra HF-V
Ra HF-V
Ra HF-V
Ra HF-V
Ra HF-V
Ra HF-V
Ra HF-V

3. FRACTIONATION OF SE ANTIGENS

1. Gel Chromatography

All SE antigens, derived from both Hl-infected and uninfected RK13 and F cells, were fractionated by gel chromatography on Sephadex G-200. Three protein peaks, designated A, B, and C were obtained in every case (figure 3). Peak B was more prominent in SE antigens H-R/SE and H-F/SE from Hl-infected cells than in control SE antigens R/SE and F/SE from uninfected cells.

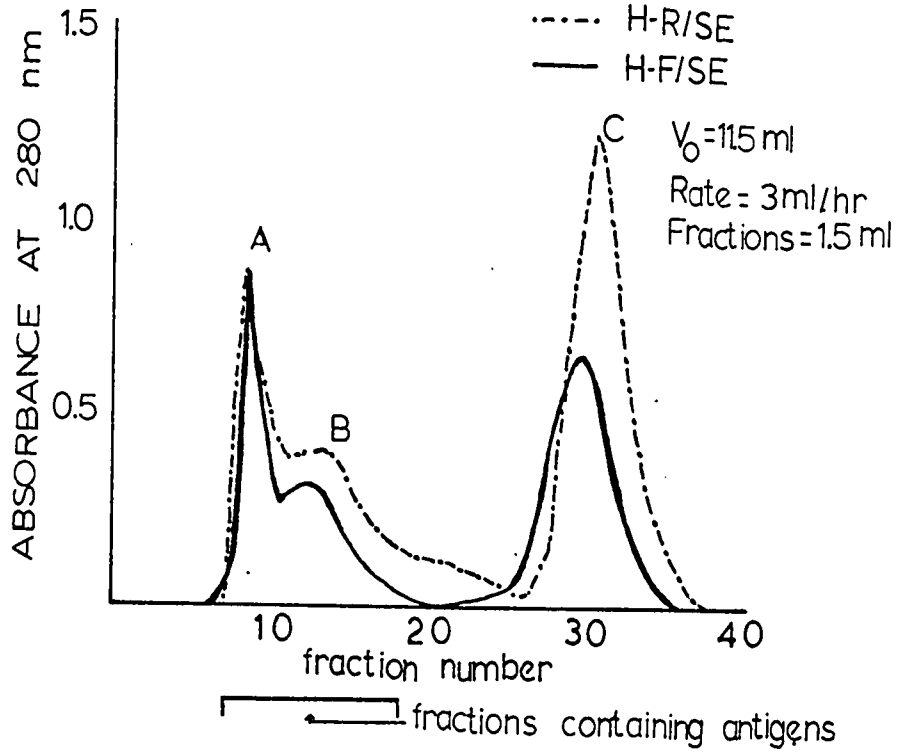
This elution profile is similar to that described by Polley and Webb (1971) for HSV antigens of infected chick embryo cells separated on Sepharose 4B agar gel columns; however, these authors did not describe a peak corresponding to Peak B.

Immunodiffusion was used to detect viral or host antigens in each fraction; those fractions in which antigens were detected are indicated at the base of the elution profile (figure 3). Fractions of Peak A were tested without concentration. Peak B fractions were divided into B1 antigens (fractions 11-14) and B2 antigens (fractions 15-18). B1 antigens, B2 antigens, and Peak C fractions 28-34 were each pooled and concentrated to the original sample volume by dialysis against 15% carboxymethylcellulose before they were tested. The immunodiffusion reactions of these antigens are illustrated in Plate 8.

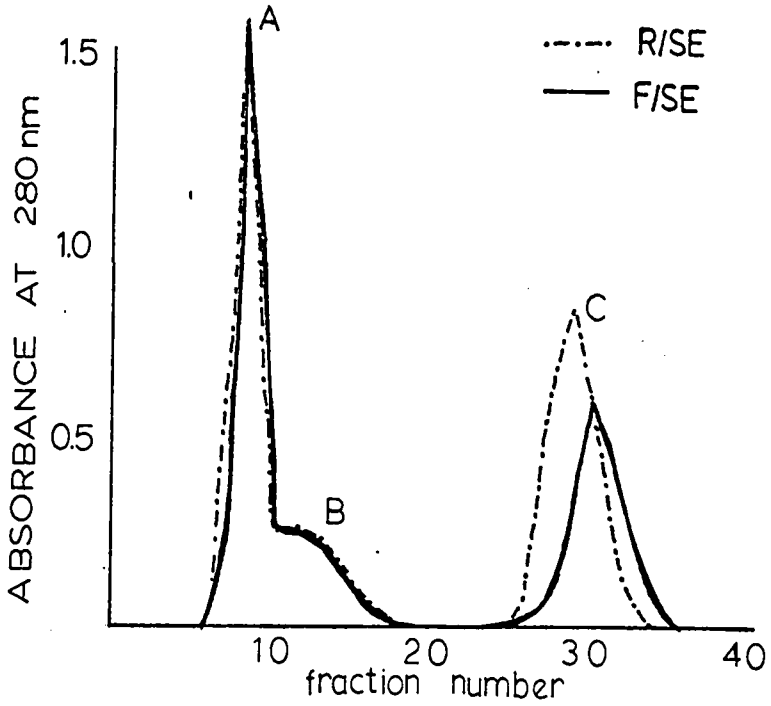
Fractions of Peak A contained host antigens, identified by their reactions with homologous but not with heterologous anti-SE sera RaHR-S or RaHF-S (figure a). These antigens, since they eluted with the void volume of the column, consisted of proteins

FIGURE 3.

Gel Chromatography of SE Antigens on Sephadex G 200



a) SE Antigens of HI infected Cells



b) SE Antigens of Uninfected Cells

PLATE 8

IDENTIFICATION BY IMMUNODIFFUSION REACTIONS OF ANTIGENIC COMPONENTS OF H-R/SE AND H-F/SE FRACTIONATED BY GEL CHROMATOGRAPHY

Legend

Antigens:

Figure a) Fractions of Peak A:
Fractions 7-10 of H-F/SE
Fractions 7-9 of H-R/SE

Figure b) Fractions of Peak B:
B1-HR-S Fractions 11-14 of H-R/SE
B2-HR-S Fractions 15-18 of H-R/SE
B1-HF-S Fractions 11-14 of H-F/SE
B2-HF-S Fractions 15-18 of H-F/SE

Antisera:

a) Rabbit:
RaHR-S to H-R/SE: soluble antigens of H1-infected RK13 cells
RaHF-S to H-F/SE: soluble antigens of H1-infected F cells

b) Human:
HuH convalescent serum

Figure a) Reactions of fractions of Peak A.
Antigenic components first appear in fraction 8, corresponding to the void volume of the column. Antigens of fractions 8, 9 and 10 react only with homologous anti-SE sera RaHR-S or RaHF-S.

Figure b) Reactions of fractions of Peak B.
At least two viral antigens are present in B1-HR-S and B1-HF-S, one of which is common to both as shown by the reaction of identity. A major viral antigen (which may be the same) is also identified in these fractions by its reaction with HuH. B2-HF-S contains at least one viral antigen reacting with RaHR-S, but B2-HR-S is not shown to contain any viral antigens in this reaction.

Ra
HFS
a
Ra
HFS
B1
HFS
B1
HFS
B1
HFS
Ra
HFS
Ra
HFS
Ra
HFS
B2
HFS
B2
HFS
Ra
HFS
Ra
HFS
b

with molecular weights around 800,000 daltons (Curling, 1970). Viral-specific proteins were expected in this region, since Keir (1968) reported that HSV-specific viral DNAase elutes largely in the void volume of Sephadex G-200. The reactions of Peak A fractions 8 and 9 of H-R/SE with homologous anti-SE serum RaHR-S is suggestive of the viral origin of these antigens, since RaHR-S did not react with host antigens of R/SE (Plate 4); but no firm identification can be made on the basis of this reaction. Failure to demonstrate viral antigens in these fractions by immunodiffusion could be due to a) their presence in amounts too small to produce visible precipitin lines with heterologous antisera or b) their denaturation or aggregation during gel chromatography, preventing their recognition by specific antibody.

Peak B1 proteins B1-HR-S and B1-HF-S contained at least one common viral antigen, identified by its immunodiffusion reactions with anti-SE serum RaHR-S (figure b). These fractions also contained a major viral antigen (which may be the same) which reacted with human immune globulin HuH. B2-HF-S also contained at least one viral antigen; B2-HR-S, on the other hand, was not shown to contain any antigens.

Peak C fractions did not react in immunodiffusion tests with any of the analytical antisera, an unexpected finding, since Polley and Webb (1971) found complement-fixing viral antigens in this region.

Viral antigens were thus identified only in fractions of Peak B; Peak A contained host (and possibly viral) antigens, but no antigens were detected in fractions of Peak C.

2. Acrylamide Gel Analytical Disc Electrophoresis

When SE antigens were fractionated by acrylamide gel disc electrophoresis, the separation patterns produced were characteristic of each host species and independent of H1 infection. Eighteen to twenty protein bands were detected in coomassie blue-stained gels of RK13-derived antigens H-R/SE and R/SE, and twenty-one to twenty-three bands in stained gels of F cell-derived SE antigens H-F/SE and F/SE. As reported by others (Watson et al, 1966; Shimono et al, 1969), no difference was apparent in the number or location of protein bands from viral-infected cells or uninfected cells by inspection of the stained gels. Immunodiffusion reactions of protein fractions of H-F/SE eluted from gel segments and tested against antiviral SE sera did, however, show that viral antigens were present in fractions 1,3,5 and 6. (Plate 9). The antigens of fraction 1 may have been present as large molecular aggregates trapped in the sample zone at the top of the gel; those of fractions 3,5 and 6 are of decreasing molecular size and increasing electrophoretic mobility.

The amounts in which these antigens were recovered in these experiments were too small to allow further comparisons with the antigens of purified virions, or with unfractionated SE antigens. Such comparisons would require the electrophoresis of these SE antigens on a preparative scale.

PLATE 9

IDENTIFICATION BY IMMUNODIFFUSION REACTIONS OF ANTIGENIC COMPONENTS OF H-F/SE FRACTIONATED BY ACRYLAMIDE GEL ELECTROPHORESIS

Legend

Antigens:

Proteins eluted from sliced gel segments after acrylamide gel electrophoresis of H-F/SE (SE antigens of Hl-infected F cells). Fraction numbers 1-11, starting at the top of the gels.

Rabbit antisera:

RaHF-S to H-F/SE
RaHR-S to H-R/SE: SE antigens of Hl-infected RK13 cells

Figure a) Reactions of fractions 1-7.
Fractions 1,3,5, and 6 contain antigenic components reacting with RaHF-S. The antigens of fractions 5 and 6 are shown to be of viral origin by their reactions with RaHR-S. The reactions of fractions 3,5, and 6 are very weak, and close to the antiserum well (arrowed).

Figure b) Reactions of fractions 5-11.
The viral origin of the antigenic component in fraction 5 is confirmed. Fractions 7-11 are not shown to contain antigens reacting with either RaHF-S or RaHR-S.

Ra HF·S
Ra HF·S
Ra HF·S
Ra HF·S
Ra HF·S
Ra HF·S
Ra HF·S
Ra HF·S
Ra HF·S
FR 1
FR 2
FR 3
FR 4
FR 5
FR 6
FR 7
Ra HR·S
Ra HR·S
Ra HR·S
Ra HR·S
Ra HR·S
Ra HR·S
Ra HR·S
Ra HR·S
Ra HR·S
a
Ra HF·S
Ra HF·S
Ra HF·S
Ra HF·S
Ra HF·S
Ra HF·S
Ra HF·S
Ra HF·S
Ra HF·S
FR 5
FR 6
FR 7
FR 8
FR 9
FR 10
FR 11
Ra HR·S
Ra HR·S
Ra HR·S
Ra HR·S
Ra HR·S
Ra HR·S
Ra HR·S
Ra HR·S
Ra HR·S
b

4. COMPARATIVE ANALYSES OF VIRAL AND HOST ANTIGENS

Comparative immunological analyses were designed to determine the origin and nature of those antigens which had been detected and characterized as described in the preceding sections. Thus, attempts were made to: 1) distinguish between host and viral antigenic components detected in purified Hl virions H-R/V and H-F/V, by means of immunodiffusion tests using adsorbed antisera, and by immuno-electron microscopy; 2) identify soluble virus structural antigens, by immunodiffusion tests comparing SE antigens with intact or disrupted virions, and by neutralization blocking tests; and 3) identify soluble viral antigens after gel chromatographic separation, by immunodiffusion comparisons with the structural antigens of intact or disrupted virions, and by neutralization blocking tests.

1. Identification of Virus Structural Components

a) Immunodiffusion Reactions with Adsorbed Antisera

Antisera, made specific for viral antigens by adsorption with normal host antigens, or made specific for host or viral non-structural antigens by adsorption with intact virions, were used to identify structural components of purified virions and SE antigens.

i) Structural Antigens of H-R/V

The viral origin of the antigenic components of H-R/V (purified Hl virions grown in RKl3 cells) detected by immunodiffusion tests (Plates 6 and 7) was confirmed by comparing the reactions of

selected antiviral sera before and after adsorption of the antisera with a) R-ex (host antigens) and b) H-R/V (purified virions). The reactions between H-R/V and these antisera, before and after adsorption, are shown in Plate 10.

One viral antigenic component of H-R/V reacted with heterologous antiviral sera RaHF-V and RaHF-S (figure a) and with convalescent rabbit serum RaHco and human serum HuH (figure b), both before and after adsorption with R-ex. Adsorption of these antisera with H-R/V, on the other hand, abolished these reactions (figures a and b).

These results confirmed earlier observations that analytical antiviral sera reacted with structural antigens of H-R/V, but not with R-C_2 host antigens in immunodiffusion tests (Plate 6). The antigenic components of H-R/V which reacted with these antisera were therefore of viral origin; unmodified host antigens, whether present as contaminants or as viral components, could not be identified. Abolishment of the immunodiffusion reactions by adsorption of these antisera with H-R/V confirmed that a specific precipitin reaction was involved, and the results could not be ascribed to non-specific precipitation of proteins.

ii) Structural Antigens of H-F/V

Immunodiffusion reactions between H-F/V (purified Hl virions grown in F cells) and homologous antisera before and after adsorption of the antisera with F-ex (host antigens) are shown in Plate 11.

PLATE 10

IDENTIFICATION OF H-R/V ANTIGENS BY IMMUNODIFFUSION REACTIONS WITH ADSORBED ANTISERA

Legend

Antigen:

H-R/V purified H1 virions grown in RK13 cells

Antisera:

a) Rabbit antisera
RaHF-V to H-F/V: purified H1 virions grown in F cells
RaHF-S to H-F/SE: SE antigens of H1-infected F cells
RaHco convalescent serum

b) Human:
HuH convalescent serum

c) Adsorbed antisera
_-aR. adsorbed with R-ex: RK13 host antigens
_-aH. adsorbed with H-R/V

Figure a) Reactions of RaHF-V and RaHF-S.
At least one viral antigen of H-R/V is detected equally well by antiviral sera RaHF-V and RaHF-S, even after adsorption with R-ex. Antisera RaHF-V-aH. and RaHF-S-aH., however, which were adsorbed with H-R/V, do not react with H-R/V.

Figure b) Reactions of RaHco and HuH.
At least one viral antigen of H-R/V is detected equally well by RaHco and HuH, even after adsorption with R-ex. RaHco-aH. and HuH-aH., however, which adsorbed with H-R/V, no longer react with H-R/V antigens.

Ra
HF-V
Ra
HF-V
aR
Ra
HF-V
aH
H-R
V
Ra
HF-S
aR
Ra
HF-S
aH
Ra
HF-S
a
Ra
Hco
Ra
Hco
aR
Ra
Hco
aH
H-R
V
Hu
H
aR
Hu
H
aH
Hu
H
b

PLATE 11

IDENTIFICATION OF H-F/V ANTIGENS BY IMMUNODIFFUSION REACTIONS WITH ADSORBED ANTISERA

Legend

Antigen:

H-F/V — purified Hl virions grown in F cells

Antisera:

a) Rabbit antisera:
RaF — to F-C_2: F cell host control antigens
RaHF-V — to H-F/V
RaHF-S — to H-F/SE: SE antigens of Hl-infected F cells

b) Adsorbed rabbit antisera:
_-aF. — adsorbed with F-ex: F cell host antigens

Figure a) Reactions with RaF, RaHF-V, and RaHF-S.
Two host antigens of H-F/V react only with unadsorbed RaF and RaHF-V, and three react only with unadsorbed RaHF-S. Two viral antigens reacting with RaHF-S, one of which also reacts with RaHF-V are identified by their reactions with viral-specific adsorbed sera. The precipitin lines between antiserum wells RaHF-S-aF., RaHF-S, and RaHF-V-aF. show that adsorption of these antisera with F-ex was complete, since excess antigens remained.

Ra
F
Ra
F
aF
Ra
HF-V
H·F
V
Ra
HF-S
aF
Ra
HF-V
aF
Ra
HF-S

Two unmodified host antigenic components of H-F/V were identified by their reactions with RaF anti-host serum, which were abolished when RaF was adsorbed with F-ex. The host origin of one of these antigenic components, as well as of one other which also reacted with anti-virion serum RaHF-V and of three which reacted with anti-SE serum RaHF-S were all confirmed when the antibodies responsible for their detection were removed by adsorption of these antisera with F-ex antigens.

Two viral antigens were identified by their reactions with adsorbed and unadsorbed RaHF-S; one of these also reacted with adsorbed and unadsorbed RaHF-V. It had been observed previously that anti-SE sera reacted with more antigenic components of purified virions than did anti-virion sera (Plate 5). Possibly SE antigens were more efficient immunizing agents than intact virions.

Two viral-specific antigenic components of H-F/V were therefore identified on the basis of these reactions. The significance of the unmodified host antigens identified could not be assessed on the basis of these results. They may have been incorporated as integral structural components of the virion; but it is more likely that they were present as contaminating material.

iii) Antigens of H-F/SE

Host and viral antigenic components of H-F/SE (SE antigens of Hl-infected F cells) were identified by immunodiffusion reactions between H-F/SE and homologous or heterologous antisera which had been adsorbed with F-ex (normal F cell antigens). These reactions are presented in Plate 12.

PLATE 12

IDENTIFICATION OF H-F/SE ANTIGENS BY IMMUNODIFFUSION REACTIONS WITH ADSORBED ANTISERA

Legend

Antigen:

H-F/SE SE antigens of Hl-infected F cells

Antisera:

RaF to F-C_2: F cell host control antigens
RaHF-V to H-F/V: purified Hl virions grown in F cells
RaHF-S to H-F/SE
RaHR-V to H-R/V: purified Hl virions grown in RKl3 cells
RaHR-S to H-R/SE: SE antigens of Hl-infected RKl3 cells
_-aF. adsorbed with F-ex: F cell host antigens

Figure a) Reactions of RaF, RaHF-V, and RaHF-S.
Four antigens detected by RaHF-S, one detected by RaHF-V and one detected by RaF are all identified as host antigens of H-F/SE because they do not react with adsorbed antisera. Three viral antigens are identified by their reactions with antiviral adsorbed serum RaHF-S-aF.; one of these also reacts with RaHF-V and adsorbed RaHF-V-aF (reactions of identity are arrowed).

Figure b) Reactions of RaHF-V, RaHR-V, and RaHR-S.
One host antigenic component of H-F/SE is identified by its reaction with homologous anti-virion serum RaHF-V, but not with adsorbed serum RaHF-V-aF., or with heterologous antiviral sera RaHR-V or RaHR-S. At least two viral antigens are identified by their reactions with heterologous adsorbed antiviral sera.

Ra F
Ra F aF
Ra HF-V
H-F SE
Ra HF-S aF
Ra HF-V aF
Ra HF-S
a
Ra HF-V
Ra HF-V aF
Ra HR-S
H-F SE
Ra HR-V aF
Ra HR-S aF
Ra HR-V
b

Four antigens reacting with homologous anti-SE serum RaHF-S, one reacting with anti-virion serum RaHF-V, and one reacting with anti-host serum RaF were identified as host components because they were not detected by the same antisera which had been adsorbed with F-ex normal cell antigens (figure a). Three viral antigenic components in H-F/SE were identified by their reactions with RaHF-S, even after adsorption of the antiserum; one of these also reacted with RaHF-V.

One viral antigenic component of H-F/SE was identified by its reactions with not only homologous anti-virion serum RaHF-V, but also with heterologous antiviral sera RaHR-V and RaHR-S, both before and after adsorption of these sera with F-ex antigens (figure b). The host component reacting with RaHF-V, shown in figure a, was also detected in this reaction.

These results confirmed earlier observations (Plates 3,4 and 5) which had suggested that some of the antigenic components of H-F/SE reacting with homologous antiviral sera RaHF-V and RaHF-S were of unmodified host origin, and permitted the identification of at least two viral components of H-F/SE.

b) Immuno-electron Microscopy of H-R/V and H-F/V

It has been previously suggested (Darlington and Moss, 1969) that unaltered host components are incorporated as integral parts of the HSV virion. Immuno-electron microscopy was used as an independent means of identifying such host components, by determining whether HI virions were agglutinated more efficiently by homologous anti-viral serum. Agglutination was quantitatively measured by counting the

numbers of free (i.e. unagglutinated) virions remaining after incubation of virus suspensions with antisera, by direct ratio to a standard number of PSL (polystyrene latex spheres). The ratios obtained for each antiserum were compared with the ratios obtained for control NRS included in each experiment, using the two-sample t-test. The results of three such experiments are shown in Table VI.

No evidence was obtained in these experiments to suggest that unmodified host components were incorporated into intact Hl virions. Anti-homologous host serum did not cause significant agglutination of either H-F/V (experiment 2) or of H-R/V (experiment 3), and adsorption of homologous antiviral sera with host antigens F-ex or R-ex did not remove viral agglutinins in either experiment. The presence of viral antigenic components in Hl virions was confirmed by the observations that heterologous antiviral sera and human serum HuH agglutinated both H-R/V and H-F/V as well as homologous antiviral sera did.

These results disagree with the conclusions of Watson and Wildy (1963b) that enveloped HSV virions were agglutinated by anti-host, but not by antiviral sera. They determined agglutination by electron microscopic observation of clumps of virions in suspensions after incubation with appropriate antiserum. Identical large clumps were seen in control suspensions in the present study, even after sonication of the suspensions for as long as six minutes, and it was for this reason that a quantitative method, which could provide an objective measurement of viral agglutination, was devised.

TABLE VI

IDENTIFICATION OF THE ANTIGENS OF H-R/V AND H-F/V BY IMMUNO-ELECTRON MICROSCOPY: DETERMINATION OF VIRAL AGGLUTINATION BY COUNTS OF RESIDUAL FREE VIRIONS AFTER INCUBATION WITH ANTISERUM

Experiment Number	Virion Antigen	Antiserum	Ratio* Free Virions / PSL spheres		Significant** Agglutination
			$\bar{x}$	$\Sigma (x-\bar{x})^2$	
1.	H-R/V	NRS control	0.89	0.2516	
		RaHR-V	0.03	0.1979	+
		RaHF-V	0.04	0.0212	+
		RaHco	0.04	0.0478	+
		HuH	0.14	0.2165	+
2.	H-F/V	NRS control	0.19	0.7896	
		RaF	0.16	0.2592	-
		RaF-aF	0.17	0.5594	-
		RaHF-V	0.02	0.0553	+
		RaHF-V-aF	0.04	0.1378	+
		RaHR-V	0.045	0.2399	+
		HuH	0.11	0.5074	+
3.	H-R/V	NRS control	0.61	1.7504	
		RaR	0.84	5.2129	-
		RaHR-V	0.13	0.7322	+
		RaHR-V-aR	0.08	0.3927	+
		RaHF-V	0.20	1.1673	+

* $\bar{x}$ = mean of 21 observations: free virions compared to numbers of PSL spheres of standard concentration 9.1×10^{10}.

$\Sigma(x-\bar{x})^2$ = variance.

** Determined by the two-sample t-test, at p=0.01, n=21.

Antiserum Abbreviations:
NRS - normal rabbit serum
HuH - human convalescent serum
RaR - rabbit serum against R-C_2 host antigens
RaF - rabbit serum against F-C_2 host antigens
RaHco - convalescent rabbit serum
RaHR-V- rabbit serum against H-R/V:purified H1 virions grown in RK13 cells
RaHF-V- rabbit serum against H-F/V:purified H1 virions grown in F cells
-aF. - adsorbed with F-ex normal F cell antigens
—aR. - adsorbed with R-ex normal RK13 cell antigens

Attempts were made to compare the numbers of free enveloped virions after incubation of virus suspensions with immune or control serum, in the hope that the presence of host antigens in the viral envelope, suggested by the work of Keller et al (1970) and of Mii et al (1968) could be confirmed by this approach. However, although results were obtained which suggested that homologous antisera agglutinated enveloped virions better than heterologous antisera did, the numbers of free enveloped virions in control suspensions were too small for statistically significant comparisons to be made, and no conclusions could be drawn from these experiments. It has been reported that the proportion of enveloped virions in suspensions of HSV depends upon the time of harvest, and upon the host cell in which the virus has replicated (Watson, 1968); possibly another host cell, such as HEp2 or Hela, would yield suspensions containing a high enough proportion of enveloped virions for a significant comparison to be made.

In these experiments, antisera were tested at only one dilution, 1:10. It would be of interest to test the effects on viral agglutination of diluting these antisera; possibly the presence of more than one viral structural antigen could be revealed by such an approach.

2. Identification of Soluble Antigens

a) Immunodiffusion Reactions of SE and Virion Antigens

Immunodiffusion tests were designed to identify the virus structural antigenic components of SE antigens by i) comparing SE

antigens with the structural antigens detectable in intact virions, ii) comparing the reactions of SE antigens with antiviral sera before and after removal of antibodies to virus structural components from the sera by adsorption, and iii) comparing the reactions of SE antigens with those of disrupted virions.

i) Comparison of H-R/SE and H-F/SE with H-R/V and H-F/V

The immunodiffusion reactions of SE and virion antigens were compared as shown in Plate 13. At least one antigenic compeneht common to F cell-derived H-F/SE soluble antigens and to H-F/V purified virions, was detected by its reactions with both homologous anti-virion serum RaHF-V and anti-SE serum RaHF-S (figure a). This antigenic component was thereby tentatively identified as a soluble virus structural antigen. Presumably it was located on the surface of the virion; no conclusion could be made on the basis of these results as to its viral or host specificity.

RK13-derived SE antigens H-R/SE and virion antigens H-R/V were compared as shown in figure b. The presence of at least one soluble virus structural antigen was indicated by a faint precipitin line between H-R/V, H-R/SE, and RaHR-S, close to the antiserum well; clear-cut reactions of identity were not, however, formed. One antigen was present in H-R/SE, but not in H-R/V, as shown by the sharp bending of the precipitin line due to this antigen away from the H-R/V antigen well. This antigen may therefore be either a) a non-structural viral antigen, or b) an internal structural

PLATE 13

IDENTIFICATION OF SOLUBLE VIRUS STRUCTURAL ANTIGENS BY COMPARATIVE IMMUNODIFFUSION OF SE AND VIRION ANTIGENS

Legend

Antigens:

H-R/SE	SE antigens of H1-infected RK13 cells
H-F/SE	SE antigens of H1-infected F cells
H-R/V	purified H1 virions grown in RK13 cells
H-F/V	purified H1 virions grown in F cells

Antisera:

RaHR-V	to H-R/V
RaHR-S	to H-R/SE
RaHF-V	to H-F/V
RaHF-S	to H-F/SE

Figure a) Reactions of H-F/SE and H-F/V.
One structural virus component of H-F/SE is identified by the reaction of identity (arrowed) between H-F/SE and H-F/V, with both RaHF-V and RaHF-S. At least one other antigenic component of H-F/V reacts with RaHF-S, and at least three other antigens of H-F/SE react with RaHF-S, and two with RaHF-V.

Figure b) Reactions of H-F/SE and H-R/V
One antigenic component of H-R/SE is not present in H-R/V, as shown by the bending of the precipitin line away from the H-R/V antigen well (arrowed). At least eight antigens of H-R/SE react with homologous anti-SE serum RaHR-S; the presence of one of these in H-R/V is indicated by a faint precipitin line (arrowed).

H-F
SE
Ra
HF-S
Ra
HF-V
H-F
V
Ra
HF-V
Ra
HF-S
H-F
SE
a
H-R
SE
Ra
HR-S
Ra
HR-V
H-R
V
Ra
HR-V
Ra
HR-S
H-R
SE
b

component which is located inside the nucleocapsid, and is therefore not detectable in intact virions.

ii) Reactions of H-R/SE with Adsorbed Antisera

The presence of structural viral components in H-R/SE (soluble antigens of H1-infected RK13 cells) was further demonstrated by the immunodiffusion reactions shown in Plate 14. H-R/SE was tested against selected antisera which had been adsorbed with R-ex (normal RK13 antigens) or H-R/V (purified RK13-grown H1 virions). Adsorption of heterologous antiviral sera RaHF-V or RaHF-S (figure a), or of convalescent rabbit serum RaHco or human serum HuH (figure b) with R-ex made no apparent difference in the reactions of these sera with H-R/SE, thus confirming previous observations that these antisera did not react with antigens of normal RK13 cells (Plates 2, 4,6), and that adsorption with R-ex did not alter their immunodiffusion characteristics (Plate 10).

Adsorption of RaHF-V and RaHF-S with H-R/V did, however, remove antibody to at least one antigen of H-R/SE and decreased the intensity of the precipitin line formed between RaHF-V and another antigenic component. (figure a). At least one, and possibly two antigenic components of H-R/SE were therefore tentatively identified, on the basis of this reaction, as virus structural components. The detection of at least one antigen was not affected by adsorption of RaHF-S with H-R/V; this antigenic component may be either an internal virus structural component, or a viral non-structural antigen.

PLATE 14

IDENTIFICATION OF VIRUS STRUCTURAL ANTIGENS OF H-R/SE
BY IMMUNODIFFUSION REACTIONS WITH ADSORBED ANTISERA

Legend

Antigen:

H-R/SE SE antigens of H1-infected RK13 cells

Antisera:

a) Hyperimmune rabbit antisera:
RaHF-V to H-F/V: purified H1 virions grown in RK13 cells
RaHF-S to SE antigens of H1-infected F cells

b) Convalescent sera:
RaHco convalescent rabbit serum
HuH convalescent human serum

c) Adsorbed antisera:
_-aR. adsorbed with R-ex: RK13 host antigens
_-aH. adsorbed with H-R/V: purified H1 virions grown in RK13 cells

Figure a) Reactions of RaHF-V and RaHF-S.
Adsorption of RaHF-V and RaHF-S with R-ex does not affect their reactions with H-R/SE; three viral antigens are detected. Antisera RaHF-V-aH and RaHF-S-aH (adsorbed with H-R/V), however, no longer react with at least one viral antigen of H-R/SE. The intensity of the reaction between a second antigen and RaHF-V is reduced when it reacts with adsorbed serum RaHF-V-aH.

Figure b) Reactions of RaHco and HuH.
Reactions between H-R/SE and RaHco are not affected by adsorption of RaHco with either R-ex or H-R/V. The reactions between HuH and H-R/SE are not affected by adsorption of HuH with R-ex; adsorption with H-R/V, on the other hand, abolishes the reaction.

Ra
HF-V
Ra
HF-V
aR
Ra
HF-V
aH
H-R
SE
Ra
HF-S
aR
Ra
HF-S
aH
Ra
HF-S
a
Ra
Hco
Ra
Hco
aR
Ra
Hco
aH
H-R
SE
Hu
H
aR
Hu
H
aH
Hu
H
b

The reactions between RaHco and H-R/SE were not affected when RaHco was adsorbed with H-R/V; but the reactions between HuH and H-R/SE were abolished by adsorption of HuH with H-R/V (figure b). These results may reflect a quantitative difference between these two antisera with respect to these particular antigenic components; or it may reflect a fundamental difference in the types and specificities of the antibodies produced by artificial infection of rabbits with H1 virus, as compared to natural recurring infection of humans.

iii) Comparison of H-R/SE with Disrupted H-R/V

The soluble antigens of H-R/SE (derived from H1-infected RK13 cells) were compared with those detectable in disrupted H-R/V (purified RK13-grown H1 virions). These immunodiffusion reactions, shown in Plate 15, confirmed earlier observations (Plate 7) that, while antigens of H-R/V were not detected after treatment with SDS, ether, or trypsin, one new antigen had been released from H-R/V by treatment with DOC. This newly-revealed antigen was shown to be identical to an antigenic component of H-R/SE detected by RaHR-V (figure a) and by RaHR-S (figure b).

Thus, disruption of H-R/V purified virions by treatment with DOC revealed the presence of another structural antigen, which was not demonstrable in immunodiffusion reactions of intact virions. A soluble antigenic component of H-R/SE was identified as a structural viral antigen by its reaction of identity with this newly-released antigen.

PLATE 15

IDENTIFICATION OF ANTIGENS OF H-R/SE BY IMMUNODIFFUSION COMPARISON WITH THOSE OF DISRUPTED H-R/V

Legend

Antigens:

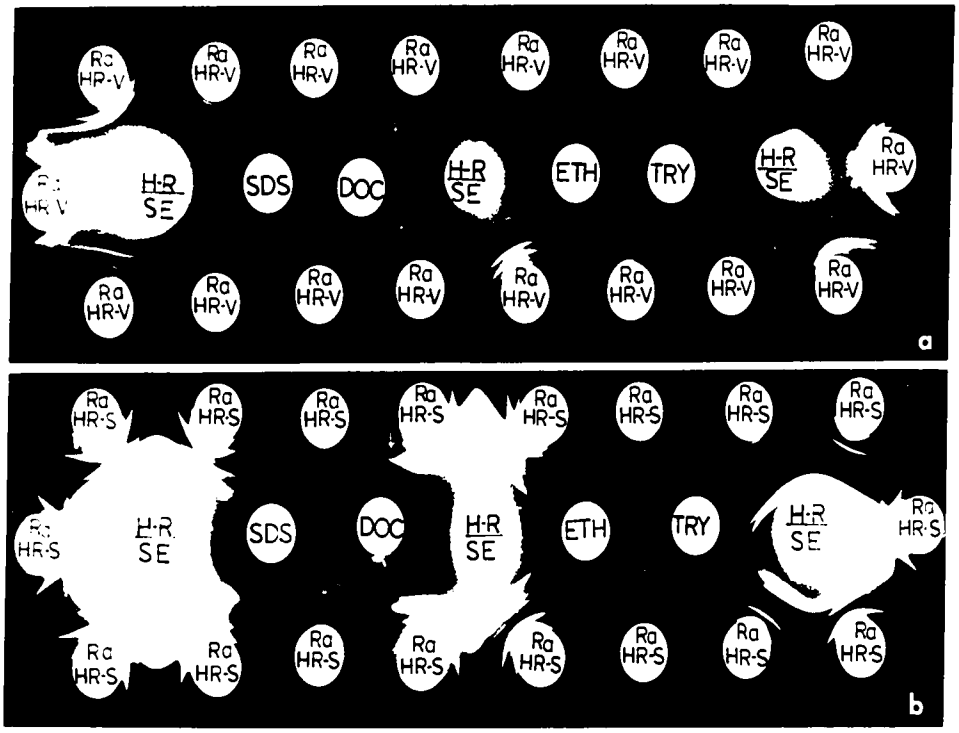
H-R/SE	SE antigens of H1-infected RK13 cells
SDS	SDS-treated H-R/V (purified H1 virions grown in RK13 cells
DOC	DOC-treated H-R/V
ETH	Ether-treated H-R/V
TRY	Trypsin-treated H-R/V

Antisera:

RaHR-V	to H-R/V
RaHR-S	to H-R/SE

Figure a) Reactions of H-R/SE and treated H-R/V with RaHR-V. Four components of H-R/SE react with RaHR-V. No antigens are detected in SDS, ETH, or TRY; but DOC has a 'new' antigen which is identical to a component of H-R/SE (reaction of identity is arrowed).

Figure b) Reactions of H-R/SE and treated H-R/V with RaHR-S. At least seven components of H-R/SE react with RaHR-S, and one of these is shown by the reaction of identity (arrowed) to be identical to a structural antigen released from H-R/V by DOC. No antigens are detected by RaHR-S in SDS, ETH, or TRY.


Ra HR-V
H-R SE
SDS
DOC
ETH
TRY
a
Ra HR-S
b

b) Neutralization Blocking Tests

Structural virus antigens, essential for infectivity, were shown to be present in SE antigens H-R/SE and H-F/SE (of H1-infected RK13 and F cells respectively) by the ability of these antigens to block neutralization of H1-R virus by homologous anti-virion serum RaHR-V, as shown in Table VII. Control SE antigens had no significant effect on the neutralization titer of RaHR-V in these experiments. A more sensitive plaque reduction test may result in the detection of blocking antigens in R/SE, but, on the basis of the results shown here, it was concluded that unmodified host components were probably not essential for virus infectivity.

3. Characterization of Soluble Antigens Fractionated by Gel Chromatography

The viral antigens separated by gel chromatography of SE antigens H-R/SE and H-F/SE on Sephadex G-200 (Plate 8) were identified by immunodiffusion and neutralization blocking tests.

a) Identification of Virus Structural Antigens by Immunodiffusion

Viral antigens which eluted in Peak B (Plate 8) were further characterized by comparing their immunodiffusion reactions with those of intact or DOC-disrupted H-R/V virions. The results, shown in Plate 16, suggested that at least one antigen, eluting in this peak and present in B2-HF-S, may be a structural component detectable in intact H-R/V virions (figure a).

Fractions of Peak B were also compared with H-R/SE antigens

TABLE VII

BLOCKING OF NEUTRALIZING ANTIBODY OF RaHR-V BY SE ANTIGENS OF Hl-INFECTED OR CONTROL CELLS

RaHR-V preincubated with:*	Neutralization Titer**	Neutralization Blocking Index (NBI)***
M199 (control titration)	640	1.0
a) SE Antigens of Hl-infected Cells:		
H-R/SE (RK13-derived)	30	0.05
H-F/SE (F-derived)	80	0.125
b) SE Antigens of Uninfected Cells:		
R/SE (RK13-derived)	560	0.85
F/SE (F-derived)	640	1.0

* RaHR-V incubated 60 minutes at 37°C with 0.8 mg protein/ml SE antigen before the addition of 250 TCD_{50} Hl-R stock virus.

** Determined in RK13 cells in micro-titer plates; reciprocal of 50% endpoint dilution calculated by the Reed-Muench method.

*** NBI = $\frac{\text{titer of test titration}}{\text{titer of control titration}}$.

Values of less than 0.25 were considered to indicate significant blocking.

PLATE 16

IDENTIFICATION OF PEAK B ANTIGENS OF H-R/SE and H-F/SE BY IMMUNODIFFUSION COMPARISON WITH INTACT AND DISRUPTED H-R/V

Legend

Antigens:

H-R/V	purified H1 virions grown in RK13 cells
H-R/SE	SE antigens of H1-infected RK13 cells
DOC	DOC-disrupted H-R/V
Fractions of Peak B:	
B1-HR-S	Fractions 11-14 of H-R/SE
B2-HR-S	Fractions 15-18 of H-R/SE
B1-HF-S	Fractions 11-14 of H-F/SE
B2-HF-S	Fractions 15-18 of H-F/SE

Antisera:

RaHR-V	to H-R/V
RaHR-S	to H-R/SE

Figure a) Reactions of H-R/V and Peak B Fractions. At least two viral antigens are identified in B1-HR-S, B1-HF-S, and B2-HF-S by their reactions with RaHR-S; one viral antigen also reacts with RaHR-V. The precipitin line (arrowed) between B2-HF-S and RaHR-S may link with that formed between H-R/V and RaHR-S. No reaction is detected between B2-HR-S and these antisera.

Figure b) Reactions of H-R/SE, DOC, and Peak B Fractions. B1-HR-S, B2-HF-S, and B1-HF-S are all shown to contain at least one viral antigen by reactions of identity (arrowed) with H-R/SE. None of these components are, however, shown to be related to the structural antigen of DOC.

Ra HR·S
B1 HFS
H·R V
B1 HR·S
Ra HR·S
Ra HR·V
Ra HR·S
B2 HFS
H·R V
B2 HR·S
Ra HR·S
Ra HR·S
a
Ra HRV
Ra HRV
Ra HR·V
Ra HR·V
Ra HR·V
Ra HR·V
Ra HR·V
H·R SE
B1 HR·S
DOC
B1 HFS
H·R SE
B2 HFS
DOC
Ra HR·S
Ra HR·S
Ra HR·S
Ra HR·S
Ra HR·S
Ra HR·S
Ra HR·S
Ra HR·S
b

and with DOC-treated H-R/V, as shown in figure b. The presence of two virus-specific soluble antigens in Peak B proteins shown previously (Plate 8) was confirmed in this reaction by their reactions of identity with whole H-R/SE produced with anti-SE serum RaHR-S. However, the reactions of identity between these fractionated antigens and DOC-disrupted H-R/V, which would have allowed their identification as virus structural antigens, were not obtained. They may, therefore be non-structural viral antigens; but such an identification must depend on the results of further analyses in which the proportions of the reactants are adequately defined and carefully controlled.

b) Neutralization Blocking Tests

Evidence that structural viral antigens were present in proteins eluting in Peak B when SE antigens H-R/SE and H-F/SE (of Hl-infected RK13 or F cells) were separated on Sephadex G-200 was obtained by the results of experiments testing the ability of these fractions to block neutralization of Hl-R virus suspensions by anti-virion serum RaHR-V. The effects of prior incubation of RaHR-V with each fraction (containing approximately 0.2 mg protein per ml) on its neutralizing titer for 250 TCD_{50} Hl-R virus are shown in Table VIII. The viral specificity of the antigens of Peak B was indicated by their significant blocking ability (NBI < 0.25). Antigens of Peaks A and C, on the other hand, had no significant effect on neutralization of Hl-R by RaHR-V.

The results of these experiments thus confirmed the presence of a virus structural antigenic component in Peak B fraction B2-HF-S,

TABLE VIII

IDENTIFICATION OF STRUCTURAL ANTIGENS IN SE FRACTIONS SEPARATED BY GEL CHROMATOGRAPHY: NEUTRALIZATION BLOCKING TESTS

RaHR-V preincubated with: *	Neutralization Titer**	Neutralization Blocking Index (NBI)***
M199 (control titration)	960	1.0
H-R/SE	40	0.04
Fractions of Peak A:		
H-R/SE fr.8	640	0.67
H-R/SE fr.9	640	0.67
Fractions of Peak B:		
B1-HR-S	160	0.16
B1-HF-S	160	0.16
B2-HR-S	640	0.67
B2-HF-S	80	0.08
Fractions of Peak C:	480	0.50

* RaHR-V incubated 60 minutes at 37°C with approximately 0.2 mg protein per ml test antigen before the addition of 250 TCD_{50}. H1-R stock virus

** Determined in RK13 cells in micro-titer plates; reciprocal of 50% endpoint dilution calculated by Reed-Muench method.

*** NBI = $\frac{\text{titer of test titration}}{\text{titer of control titration.}}$ Values of less than 0.25 indicate significant blocking.

which was suggested by the immunodiffusion results shown in Plate 16; and furthermore, they indicated the presence of structural components in Peak B fractions B1-HR-S and B1-HF-S which were not demonstrated by immunodiffusion tests. The number of these structural components cannot, of course, be determined from these results. Viral antigens were not demonstrated either by these results, or by immunodiffusion, in fractions B2-HR-S, or in fractions of Peaks A and C.

5. SUMMARY OF EXPERIMENTAL RESULTS

The antigens produced in cells of two host species (RK13 cells of rabbit origin and F cells of chick embryo origin) as a result of infection with the H1 (type 1) strain of HSV were characterized using a battery of rabbit and human analytical antisera. Soluble (SE) and purified virion (V) antigens were prepared from H1-infected cells of each host, and characterized by comparison with each other and with control antigens of uninfected host cells, using immunodiffusion, neutralization blocking, and immuno-electron microscopy. SE antigens were further characterized by gel chromatography and acrylamide gel electrophoresis.

Cellulose acetate membranes, used for immunodiffusion studies, allowed the diffusion of intact virions, and permitted better separation and resolution of precipitin lines of complex reactions than did standard agarose support medium.

An improved method was devised for the quantitative estimation of viral agglutination, based on electron microscopic particle counts of residual free unagglutinated virions. The significance of the results obtained was confirmed by statistical methods.

Eight antigenic components of SE antigens and two of purified virions were identified as viral antigens by their reactions with hyperimmune rabbit antisera. Two of these soluble antigens, and one virion antigen also reacted with human convalescent sera, and were thus identified as major viral antigens, responded to during human

infection, and common to more than one strain of HSV.

An internal virion antigenic component, released when purified virions were disrupted with DOC, reacted with hyperimmune rabbit antiviral sera.

Three unmodified host components of SE antigens, and two of purified virion suspensions were detected in viral antigens derived from H1-infected F cells. No evidence was obtained by immuno-electron microscopy or by neutralization blocking experiments to suggest that these unmodified host antigens were incorporated into H1 virions. Unmodified host antigens were not detected in RK13-derived antigens; but the presence of a viral-modified host antigen in suspensions of purified H1 virions grown in RK13 cells was suggested by its reaction with anti-host serum in immunodiffusion tests.

Gel chromatography on Sephadex G-200 resulted in the characterization of at least three viral antigens which eluted in the same protein peak. One of these was proven to be a virus structural component by immunodiffusion reactions with intact and DOC-disrupted virions. Neutralization blocking tests provided evidence that other structural antigens were present as well.

V. DISCUSSION

DISCUSSION

The immediate objectives of this investigation were threefold: first, to demonstrate the complete spectrum of antigens in HSV-infected cells; second, to characterize these as normal host antigens, as viral antigens, or as antigens with dual host-virus specificity; and third, to distinguish between structural antigenic components of the virion and viral non-structural antigens involved in viral replication.

Immunodiffusion, neutralization blocking, and immuno-electron microscopy were the three methods chosen for the accomplishment of these objectives.

Previous immunodiffusion studies of HSV antigens have used lyzed infected cells as the source of viral antigens (Watson et al, 1966; Tokumaru, 1970). Immunodiffusion studies of purified virions have not been possible, since HSV virions (diameter 100-180 nm) are too large to diffuse through 1% agar gels, whose pore size has been calculated to be 60-100 nm (Ackers and Steere, 1962). The introduction of cellulose acetate to immunodiffusion studies of HSV antigens has made possible a) the detection of at least two virion-associated antigens which diffuse in this medium but not in agar, and b) the identification of soluble viral antigenic components by reactions of identity which formed in cellulose acetate but not in agar.

The existence of extremely labile viral antigens, which disappeared in 48 hours at 4°C, in extracts of HSV-infected cells

has recently been reported (Tokumaru, 1970; Tarro and Sabin, 1970). The detection of such labile antigens was not attempted, and it is not likely that they were among the antigens recognized in the present study. Only relatively stable antigens were characterized, since storage at 4° C for as long as two or three weeks did not affect their immunodiffusion or neutralization blocking reactions.

The identification and characterization of any antigen depends absolutely on the properties of the antisera used for its recognition. The use of antisera whose characteristics were ill-defined has resulted in some confusion in the literature (Nii et al, 1968). An attempt was made to overcome this problem by the use of a battery of analytical antisera. Hyperimmune antisera produced in rabbits in response to immunization with antigens whose source and characteristics were known were used in conjunction with antiviral sera produced as a result of artificial infection of rabbits or of natural recurring infection of humans. As expected, more antigens were detected by hyperimmune sera than by convalescent sera; but the use of convalescent human serum permitted the tentative identification of two major virus antigens, shared by several strains of HSV and which are immunogenic in natural human infection.

The identification of antigens of host origin was made possible in this study by the use of two antigenically-unrelated host systems, the RK13 cell line of rabbit origin, and F cells derived from chick embryos. Antigens produced in each species of host cell could then be compared, using antisera prepared against

antigens derived from cells either homologous or heterologous to the host cell from which test viral antigens were produced.

Antibody to host antigens of RK13 cells was not detectable in the sera of rabbits immunized with RK13-derived host control or viral antigens. Reactions due to unmodified host antigens were therefore eliminated from the test system when RK13-derived antigens were tested by immunodiffusion against homologous analytical antisera. One antigenic component of purified H1 virions grown in RK13 cells was, however, shown to react with anti-RK13 host serum. This reaction could have been due to a) the presence of a host cell contaminant, b) selective incorporation into virions of a normal cell component which was present, but below detectable levels in the control antigens tested, or c) incorporation into virions of a viral-modified host component which was not reactive in its unaltered state with anti-host serum, thus resulting in a new antigen with dual host-virus specificity. Evidence supporting this third possibility is provided by the results of Keller et al (1970) who showed that viral-altered cell membranes are incorporated into HSV virions.

The presence of unaltered host antigens of F cells in viral antigens produced in these cells was shown by immunodiffusion tests against homologous antisera. However, as is the case with other enveloped virions, such as those of the Myxovirus group (Dimmock and Watson, 1969) the detection of host antigens in purified virion suspensions does not mean that they are integral components of infectious virions, since host components may be present simply as

impurities. The virus purification procedures used here were not exhaustive, and consisted simply of differential and sucrose density gradient centrifugation. Thus, conclusions as to the form in which host antigens were present cannot be drawn on the basis of these immunodiffusion results.

An attempt was made to overcome this difficulty by the development of immuno-electron microscopic techniques. The presence of unmodified host structural components in intact virions could be confirmed if virions were agglutinated by homologous anti-host or antiviral sera, but not by heterologous antiviral serum. No evidence for the presence of such host components was obtained; heterologous antisera agglutinated virions as well as homologous sera did, and adsorption of homologous antisera with host antigens did not affect their agglutinating potency. Thus, in contradiction to previous authors (Watson and Wildy, 1963b), it was concluded that unaltered host antigens were not present in HSV virions.

The treatment of intact virions with disrupting agents in order to release previously-hidden internal antigens which could then be detected in immunodiffusion reactions was used by Westwood et al (1965) for the identification of vaccinia virus structural antigens. Soluble virus structural antigens were identified by reactions of identity with newly-released antigens of disrupted virions

in immunodiffusion tests. Such studies on the structural antigens of HSV have not been reported before. Four disrupting agents, SDS, DOC, ether and trypsin were investigated, and DOC was shown to release a newly-detectable structural antigen, which presumably had formerly been hidden inside the nucleocapsid. Both SDS and DOC solubilized virus suspensions completely; treatment with ether or trypsin, although destroying infectivity, neither released new antigens, nor could be seen by electron microscopy to affect viral morphology.

Fractionation of soluble SE antigens on Sephadex G-200 resulted in the separation of three protein peaks, only one of which, peak B, was shown to contain viral antigens by immunodiffusion and neutralization blocking tests. Failure to demonstrate viral antigens in peaks A and C was an unexpected finding, since HSV-induced enzymes were reported to elute, like peak A, in the void volume of Sephadex G-200. (Keir, 1968). It is possible that these proteins, although present, were aggregated, and no longer recognizable as antigens by the analytical antisera.

Failure to distinguish between SE antigens derived from Hl-infected or uninfected cells by visual examination of stained gels after acrylamide gel electrophoresis is in accord with results reported by other authors (Watson et al, 1966; Shimono et al, 1969), although radioactive labelling techniques have proven successful in identifying viral proteins (Spear et al, 1968; Olshevsky et al, 1970). The immunological techniques used in the present study were powerful

enough to detect and identify at least three antigenic components after electrophoresis of the SE antigens of Hl-infected cells. The fact that these were antigenic implies that they possessed a functional significance in infection which cannot be inferred from comparable results obtained by biochemical labelling techniques alone. However, the amounts of these antigenic components recovered from gel segments in this semi-micro technique were insufficient to allow any comparisons of these separated components with antigens of virion or complete SE antigen preparations; such comparisons require amounts of antigens that can only be obtained by acrylamide gel electrophoresis on a preparative scale.

At least one viral antigen, which eluted with Peak B antigens from Sephadex G-200, was shown to be a structural virus component by its reaction of identity with an antigens of DOC-disrupted Hl virions in immunodiffusion tests. This antigen may be identical to a soluble structural antigen described by Tokumaru (1965), of molecular weight 500,000, and thought by him to consist of viral capsomeres. The presence of at least one, and possibly more, other structural antigens in Peak B fractions was indicated by the results of neutralization blocking tests, which showed that these fractions blocked neutralization of virus by specific antibody, and reacted with antiviral sera in immunodiffusion, even though they could not be shown to be identical to Hl virion structural antigens.

It is clear that the results presented in this study are only preliminary steps in the characterization of the antigens of HSV. Further experiments, using the methods described here, are needed for a more complete identification of virus structural and non-structural soluble antigens, and of the postulated host component of the HSV envelope and/or nucleocapsid. The results of such studies may be of value in determining the significance of these antigenic components in viral replication and/or in eliciting or modifying normal host responses in such a way as to establish the persistent infections which are so characteristic of members of the herpesvirus group.

VI. REFERENCES

REFERENCES

Ackers,G.K. and Steere,R.L. 1962. Restricted diffusion of macromolecules through agar gel membranes. Biochim.Biophys. Acta 59: 137-149.

Asher,Y., Heller,M. and Becker,Y. 1969. Incorporation of lipids into herpes simplex virus particles. J. gen. Virol. 4: 65-76.

Beale,A.J., Christofinis,G.C. and Furminger,I.G.S. 1963. Rabbit cells susceptible to rubella virus. Lancet 285: 640-641.

Becker,Y., Dym,H. and Sarov,I. 1968. Herpes simplex virus DNA. Virology 36: 184-192.

Becker,Y., Olshevsky,U. and Levitt,J. 1967. The role of arginine in the replication of herpes simplex virus. J. gen. Virol. 1: 471-478.

Brown, A. and Officer,J.E. 1968. Contamination of cell cultures by Mycoplasma (PPLO). In Methods in Virology, vol. IV. Edited by K. Maramorosch and H.M. Koprowski. Academic Press, New York: 531-561.

Buchan,A. and Watson,D.H. 1969. The immunological specificity of thymidine kinases in cells infected by viruses of the herpes group. J. gen. Virol. 4: 461-463.

Cliver,D.O. 1968. Virus interactions with membrane filters. Biotech. and Bioeng. 10: 877-889.

Courtney,R.J., McCombs,R.M. and Benyesh-Melnick,M. 1970. Antigens specified by herpesviruses. I. Effect of arginine deprivation on antigen synthesis. Virology 40: 379-386.

Crowle,A.J. 1958. A simplified micro-double diffusion agar precipitin technique. J. Lab. Clin. Med. 52: 784-787.

Curling,J. 1970. The use of Sephadex in the separation, purification and characterization of biological materials. In Experiments in Physiology and Biochemistry, vol. 3. Edited by G.A. Kerkut. Academic Press, New York: 417-484.

Darlington,R.W. and Moss,L.H. 1969. The envelope of herpesvirus. Progr. Med. Virol. 11: 16-45.

Davis,B.J. and Ornstein,L. 1968. Disc electrophoresis in acrylamide gel columns. In Methods in Immunology and Immunochemistry, vol. II. Edited by C.A. Williams and M.W. Chase. Academic Press, New York: 38-47.

Dimmock,N.J. and Watson,D.H. 1969. Proteins specified by influenza virus in infected cells. Analysis by polyacrylamide gel electrophoresis of antigens not present in the virus particle. J. gen. Virol. 5: 499-509.

Dowdle,W.R., Nahmias,A.J., Harwell,R.W. and Pauls,F.P. 1967. Association of antigenic type of herpesvirus hominis with site of viral recovery. J. Immunol. 99: 974-980.

Dubbs,D.R. and Kit,S. 1964. Mutant strains of herpes simplex deficient in thymidine kinase-inducing activity. Virology 22: 493-502.

Fenner,F. 1970. The genetics of animal viruses. Ann. Rev. Microbiol. 24: 297-334.

Fuccillo,D.A., Moder,F.A., Catalano,L.W., Vincent,M.M. and Sever,J.L. 1970. Herpesvirus hominis types 1 and 2: A specific micro-indirect hemagglutination test. Proc. Soc. Exptl. Biol. Med. 133: 735-739.

Ginsberg,H.S. 1969. Biochemistry of adenovirus infection. In Biochemistry of Viruses. Edited by H.B. Levy. Marcel Dekker, New York: 329-359.

Ginsberg,H.S., Pereira,H.G., Valentine,R.C. and Wilcox,W.C. 1966. A proposed terminology for the adenovirus antigens and virion morphological subunits. Virology 28: 782-783.

Gold,E., Wildy,P. and Watson,D.H. 1963. The development of infectivity, antigens and particles in herpes infected cells. J. Immunol. 91: 666-669.

Hayflick,L. and Stanbridge,E. 1967. Isolation and identification of mycoplasma from human clinical materials. Ann. N.Y. Acad. Sci. 143: 608-621.

Hayward,M.E. 1950. Serological diagnosis of herpes simplex infections. Lancet 258: 856.

Henle,G., Henle,W. and Diehl,V. 1968. Relation of Burkitt's tumor-associated herpes-type virus to infectious mononucleosis. Proc. Natn. Acad. Sci. U.S.A. 59: 94-101.

Hsiung,G.D. 1964. Diagnostic Virology. Yale University Press, New York.

Johnson,C.M., Westwood, J.C.N.,and Beaulieu,M. 1964. A continuous flow micro-double diffusion technique in cellulose acetate. Nature 204: 1321-1322.

Johnson,C.M. and Westwood,J.C.N. 1971. Immunodiffusion studies on the myxoviruses. III. Identification of the structural antigens of myxovirus influenza A/PR8. Can. J. Microbiol. 17: 915-920.

Josey,W.F., Nahmias,A.J. and Naib,Z.M. 1968. Genital infection with type 2 herpesvirus hominis. Present knowledge and possible relation to cervical cancer. Amer. J. Obst. Gynec. 101: 718-729.

Keir,H.M. 1968. Virus-induced enzymes in mammalian cells infected with DNA viruses. Symp. Soc. Gen. Microbiol. XVIII: 67-99.

Keir,H.M. and Gold,E. 1963. Deoxyribonucleic acid nucleotidyltransferase and deoxyribonuclease from cultured cells infected with herpes simplex virus. Biochim. Biophys. Acta 72: 263-276.

Keller,J.M., Spear,P.G. and Roizman,B. 1970. Proteins specified by herpes simplex virus. III. Viruses differing in their effects on the social behaviour of infected cells specify different membrane glycoproteins. Proc. Natn. Acad. Sci. U.S.A. 65: 865-871.

Kit,S., Dubbs,D.R. and Anken,M. 1967. Altered properties of thymidine kinase after infection of mouse fibroblast cells with herpes simplex virus. J. Virol. 1: 238-240.

Kleger,B. and Prier,J.E. 1969. Herpes simplex infection of the female genital tract. II. Association of cytopathic effect in cell culture with site of infection. J. Inf. Dis. 120: 376-378.

Klemperer,H.G., Haynes,G.R., Shedden,W.I.H. and Watson,D.H. 1967. A virus-specific thymidine kinase in BHK21 cells infected with herpes simplex virus. Virology 31: 120-128.

Legget Bailey,J. 1962. Techniques in Protein Chemistry. Elsevier Publishing Co., New York.

Miyamoto,K., Morgan,C., Hsu,K.C. and Hampar,B. 1971. Differentiation by immunoferritin of herpes simplex virion antigens with the use of rabbit 7S and 19S antibodies from early (7-day) and late (7-week) immune sera. J. Nat. Canc. Inst. 46: 629-646.

Nahmias,A.J., Chiang,W.T., DelBuono,I. and Duffey,A. 1969. Typing of herpesvirus hominis by a direct immunofluorescent technique. Proc. Soc. Exptl. Biol. Med. 132: 386-390.

Nahmias,A.J. and Dowdle,W.R. 1968. Antigenic and biologic differences in herpesvirus hominis. Progr. Med. Virol. 10: 110-159.

Nii,S., Morgan,C., Rose,H.M. and Hsu,K.C. 1968. Electron microscopy of herpes simplex virus. IV. Studies with ferritin-conjugated antibodies. J. Virol. 2: 1172-1184.

Olshevsky,U. and Becker,Y. 1970. Herpes simplex virus structural proteins. Virology 40: 948-960.

Peutherer,J.F. 1970. The specificity of rabbit antisera to herpesvirus hominis and its dependence on the dose of virus inoculated. J. Med. Microbiol. 3: 267-272.

Plummer,G., Waner,J.L., Phuangsab,A. and Goodheart,C.R. 1970. Type 1 and type 2 herpes simplex viruses: serological and biological differences. J. Virol. 5: 51-59.

Polley,J.R. and Webb,T.S. 1971. Comparison of herpes simplex antigens prepared from various tissues. Can. J. Microbiol. 17: 821-822.

Rawls,W.E., Tompkins,W.A.F., Figueroa,M.E. and Melnick,J.L. 1968. Herpesvirus type 2: association with carcinoma of the cervix. Science 161: 1255-1256.

Rawls,W.E., Tompkins,W.A.F. and Melnick,J.L. 1969. The association of herpesvirus type 2 and carcinoma of the uterine cervix. Amer. J. Epidemiol. 89: 547-554.

Roane,P.R. and Roizman,B. 1964. Studies on the determinant antigens of viable cells. II. Demonstration of altered antigenic reactivity of HEp2 cells infected with herpes simplex virus. Virology 22: 1-8.

Robinson,D.J. and Watson,D.H. 1971. Structural proteins of herpes simplex virus. J. gen. Virol. 10: 163-171.

Roeppnack,G. 1965. Sodium dodecyl sulfate purification for nucleic acid isolation. Ger. (east) Patent 39, 209. Chem. Abstr. 63: 18488a.

Roizman,B. 1969. Herpesviruses. In Biochemistry of Viruses. Edited by H.B. Levy. Marcel Dekker, New York: 415-482.

Roizman,B., Spring,S.B., and Roane,P.R. 1967. Cellular compartmentalization of herpesvirus antigens during viral replication. J. Virol. 1: 181-192.

Rosenbaum,M.J., Edwards,E.A. and Sullivan,E. 1970. Micromethods for respiratory virus sero-epidemiology. Health Lab. Sci. 7: 42-52.

Ross,L.J.N., Watson,D.H. and Wildy,P. 1968. Development and localization of virus-specific antigens during multiplication of herpes simplex virus in BHK21 cells. J. gen. Virol. 2: 115-122.

Russell,W.C., Watson,D.H. and Wildy,P. 1963. Preliminary chemical studies on herpes virus. Biochem. J. 87: 26p-27p.

Scholtissek,C., Drzeniek,R. and Rott,R. 1969. Myxoviruses. In Biochemistry of Viruses. Edited by H.B. Levy. Marcel Dekker, New York: 220-258.

Schwartz,J. and Roizman,B. 1969. Concerning the egress of herpes simplex virus from infected cells: electron and light microscopic observations. Virology 38: 42-49.

Shimono,H., Ben-Porat,T. and Kaplan,A.S. 1969. Synthesis of proteins in cells infected with herpesvirus. I. Structural viral proteins. Virology 37: 49-55.

Shipkey,F.H., Erlandson,R.A., Bailey,R.B., Babcock,V.I. and Southam, C.M. 1967. Virus biographies. II: Growth of herpes simplex virus in tissue culture. Exp. Molec. Pathol. 6: 39-67.

Spear,P.G., Keller,J.M. and Roizman,B. 1970. Proteins specified by herpes simplex virus. II: Viral glycoproteins associated with cellular membranes. J. Virol. 5: 123-131.

Spear,P.G. and Roizman,B. 1967. Buoyant density of herpes simplex virus in solutions of cesium chloride. Nature 214: 713-714.

Spear,P.G. and Roizman,B. 1968. The proteins specified by herpes simplex virus. Time of synthesis, transfer into nuclei and properties of proteins made in productively infected cells. Virology 36: 545-555.

Spear,P.G. and Roizman,B. 1970. Proteins specified by herpes simplex virus. IV: Site of glycosylation and accumulation of viral membrane proteins. Proc. Natn. Acad. Sci. U.S.A. 66: 730-737.

Stewart,S.E. 1969. Studies on the herpes-type virus recovered from the Burkitt's tumor and other human lymphomas, Adv. Vir. Res. 15: 291-305.

Tarro,G. and Sabin,A.B. 1970. Virus-specific labile non-virion antigens in herpesvirus-infected cells. Proc. Natn. Acad. Sci. U.S.A. 65: 753-760.

Tokumaru,T. 1965. Studies of herpes simplex virus by the gel diffusion technique. II: The characterization of viral and soluble precipitating antigens. J. Immunol. 95: 189-195.

Tokumaru,T. 1969. Herpesviruses. In Diagnostic Procedures for Viral and Rickettsial Infections. Edited by E.H. Lennette and N.J. Schmidt. American Public Health Association, New York: 641-700.

Tokumaru,T. 1970. Further analysis of physicochemical and immunologic properties of herpes simplex virus precipitating antigens. Arch. ges. Virusforsch. 29: 295-306.

Watkins, J.F. 1965. The relationship of the herpes simplex hemadsorption phenomenon to the virus growth cycle. Virology 26: 746-753.

Watson,D.H. 1968. The structure of animal viruses in relation to their biological functions. Symp. Soc. Gen. Microbiol. XVIII: 207-228.

Watson,D.H. 1969. The separation of herpes-virus-specific antigens by polyacrylamide gel electrophoresis. J. gen. Virol. 4: 151-161.

Watson,D.H., Shedden,W.I.H., Elliot,A., Tetsuka,T., Wildy,P., Bourgaux-Ramoisy,D. and Gold,E. 1966. Virus specific antigens in mammalian cells infected with herpes simplex virus. Immunology 11: 399-408.

Watson,D.H. and Wildy,P. 1963a. Electron microscopic particle counts on herpes virus using the phosphotungstate negative staining technique. Virology 19: 250-260.

Watson,D.H. and Wildy,P. 1963b. Some serologic properties of herpes virus particles studied with the electron microscope. Virology 21: 100-111.

Watson,D.H. and Wildy,P. 1969. The preparation of monospecific antisera to herpes virus specific antigens. J. gen. Virol. 4: 163-168.

Watson,D.H., Wildy,P., Harvey,B.A. and Shedden,W.I.H. 1967. Serological relationships among viruses of the herpes group. J. gen. Virol. 1: 139-141.

Westwood,J.C.N., Zwartouw,H.T., Appleyard,G. and Titmuss,D.H.J. 1965. Comparison of the soluble antigens and virus particle antigens of vaccinia virus. J. gen. Microbiol. 38: 47-53.

Wheeler,C.E., Briggaman,R.A. and Henderson,R.R. 1969. Discrimination between two strains (types) of herpes simplex virus by various modifications of the neutralization test. J. Immunol. 102: 1179-1192.

Wildy,P. and Holden,H.F. 1954. The complement-fixing antigen of herpes simplex virus. Austr. J. Exp. Biol. Med. Sci. 32: 621-632.

Wildy,P., Russell,W.C. and Horne,R.W. 1960. The morphology of herpes virus. Virology 12: 204-222.

VII. APPENDIX

APPENDIX

TABLE OF CONTENTS

I. DETECTION OF PPLO (MYCOPLASMA) CONTAMINATION OF RK13 CELL CULTURES

RK13 cell cultures were monitored at regular intervals for PPLO contamination, using the procedures outlined by Brown and Officer (1968). Spent medium and trypsinized cell suspensions were sampled during regular cell passages, and 0.2 ml of each inoculated into each of two tubes of PPLO broth (Difco), supplemented with 20% agamma horse serum (Gibco), 0.5% Difco yeast extract and 100 units/ml penicillin and 100 ug/ml streptomycin. One tube was incubated aerobically at 37°C, and the other anaerobically, in a mixture of 95% nitrogen-5% CO_2. At intervals of 2, 5 and 14 days, each broth was subcultured onto Difco PPLO agar containing 10% agamma horse serum and 1% yeast extract in 60 mm plastic petri plates. The plates were incubated at 37°C aerobically and anaerobically, and examined microscopically after 2 and 5 days for the appearance of typical PPLO colonies.

II. THE PREVENTION OF PPLO CONTAMINATION BY THE USE OF AUREOMYCIN

The incorporation of aureomycin into the culture medium of serial cell lines has been found to be an effective means of preventing PPLO contamination (Hayflick and Stanbridge, 1967). Its effectiveness for the prevention of infection of RK13 cells by a strain of PPLO isolated in this laboratory was therefore investigated.

1. PPLO Strain H-PR7

PPLO strain H-PR7 was isolated from an RK13 cell culture early in the course of this work. It produced diffuse turbidity in PPLO broth culture, and typical 'fried egg' colonies about 1 mm in diameter after 48 hours incubation on PPLO agar. It grew well aerobically.

Viable plate counts of 48-hour broth cultures of H-PR7 were performed by making serial tenfold dilutions of the culture and plating 0.1 ml of appropriate dilutions of the surface of PPLO agar plates. The plates were examined after 48 hours incubation, and the numbers of colonies counted.

2. Determination of the Cytotoxic Level of Aureomycin

Doubling dilutions of a stock solution of aureomycin (5000 ug/ml) were made in 0.5 ml volumes of M199 in serological tubes, 0.5 ml RK13 cells ($1x10^5$ cells) in growth medium added, and 0.2 ml from each tube seeded into each of four wells of a micro-titer plate. The cultures were incubated at 37°C in a candle

jar, and examined daily. Cytotoxicity was evident by rounding and clumping of the cells, with failure to attach to the plastic.

The results showed that 50 ug/ml aureomycin was not cytotoxic for RK13 cells. Therefore, this level of aureomycin, incorporated into the growth medium, was investigated for the prevention of PPLO infection.

3. Prevention of PPLO Infection with Aureomycin

Two groups of nine plastic tissue culture 30 ml flasks (Falcon Plastics Ltd.) were seeded with 4 ml RK13 cells ($2x10^5$ cells/ml) in growth medium. Group I contained aureomycin 50 ug/ml in the medium, and Group II contained 100 u penicillin and 100 ug streptomycin per ml. The medium was changed after five days incubation. On the sixth day, six cultures of each group were inoculated with 0.1 ml of a saline suspension of P-HR7, which contained approximately 100 organisms, confirmed by viable plate counts. The remaining three cultures in each group served as controls, and were inoculated with 0.1 ml saline.

Five days later, each culture was tested for the growth of PPLO by inoculating 0.2 ml medium into PPLO broth, subculturing the broth after 48 hours onto PPLO agar, and examining the agar plates for typical PPLO colonies after 48 hours incubation.

The results, which are summarized in Table A-I, showed that 50 ug/ml aureomycin incorporated into the culture medium was effective in preventing the establishment of infection by the P-HR7 strain of PPLO, even when a fairly large number of viable organisms was

deliberately introduced.

Accordingly, the use of aureomycin was adopted as a routine procedure in the culture of RK13 cells for the production of PPLO-free viral and control antigens.

TABLE A-I

PREVENTION OF P-HR7 (PPLO) INFECTION OF RK13 CELL CULTURES BY THE USE OF AUREOMYCIN

Number of RK13 cell cultures infected: *

	Group I	Group II
Antibiotic	aureomycin 50 ug/ml	penicillin-streptomycin 100 u-ug/ml
Inoculated with:		
0.1 ml P-HR7 (100 viable organisms)	0/6	6/6
0.1 ml saline	0/3	0/3

* Determined by isolation of PPLO five days after inoculation

III. INFECTIVITY ASSAYS

Comparison of the micro-titer plate and standard tube methods

RK13 cells in growth medium were dispensed in 1 ml volumes into rubber-stoppered test tubes, and incubated 4 days at 37°C in a slanted position. When a monolayer had formed, the medium was replaced with growth medium in which the concentration of fetal calf serum had been reduced to 2%, and the cells used for comparative infectivity titrations.

Serial tenfold dilutions of two H1 virus suspensions (one, H1-R was done in duplicate) were made in 1 ml volumes of M199 in serological tubes; 0.1 ml of each virus dilution was inoculated into each of four tube cultures, and 0.5 ml added to 0.5 ml RK13 cells ($1x10^5$ cells) in growth medium. After mixing, 0.2 ml virus-cell mixture was inoculated into each of four wells of a plastic micro-titer plate. The inoculated cell cultures were incubated at 37°C; plate cultures were placed in a candle jar. All cultures were examined daily for the presence of viral CPE. Titers were calculated by the Reed-Muench method, and expressed as TCD_{50}/ml. The final titer, and the number of days required to attain a final reading by each method are summarized in Table A-II.

The results showed that the two methods were comparable in their sensitivity; slightly lower titers were recorded in micro-titer plates, but the maximum titer was recorded in only 2-3 days, as compared to 5-7 days required by the standard tube method.

TABLE A-II

INFECTIVITY TITRATIONS OF H1 VIRUS SUSPENSIONS: COMPARISON OF RESULTS OBTAINED BY THE STANDARD TUBE METHOD WITH THOSE OBTAINED BY THE MICRO-TITER PLATE METHOD.

Virus suspension	METHOD			
	Tube		Micro-titer plate	
	* Titer	** Days	Titer	Days
*** H1-R	$10^{7.3}$	5	$10^{7.0}$	2
H1-R	$10^{7.5}$	7	$10^{7.5}$	3
H1-F	$10^{5.8}$	5	$10^{5.5}$	2

* Expressed as TCD_{50}/ml., calculated by the Reed-Muench method.

** Number of days required to attain maximum titer.

*** H1-R virus suspension was assayed in duplicate.

IV. IMMUNOELECTROPHORESIS OF SE ANTIGENS

Immunoelectrophoresis of SE antigens H-R/SE and H-F/SE was investigated as a means of separating and characterizing the antigenic components of these mixtures.

Glass slides (1 x 3 ") were coated with 2% agarose in barbital buffer (Buchler) pH 8.6, u 0.075, containing 0.1% NaN_3, and stored 2-24 hours in a moist chamber. After wells were cut in the agar, the slides were placed in a electrophoretic chamber filled with the same buffer and connected to a constant voltage power supply set at 200 v. After the slides were pre-run 60 minutes, each well was filled with 0.05 ml H-R/SE or H-F/SE containing 8 mg protein per ml, and electrophoresis continued 60-80 minutes. Troughs were then cut and charged with antiserum, and the slides placed in a moist chamber at 22°C for 24-48 hours. After precipitation lines had formed, the slides were dried, stained with thiazine red (Crowle,1958) and photographed.

Separation of H-R/SE and H-F/SE by immunoelectrophoresis resulted in the detection of only one viral antigen, identified by its reaction with both homologous and heterologous anti-virion sera RaHR-V and RaHF-V (Plate A-I). On the basis of this result, it was concluded that immunoelectrophoresis was not of particular value in this study, and its use was not pursued further.

PLATE A-I

IMMUNOELECTROPHORESIS OF SE ANTIGENS H-R/SE AND H-F/SE

LEGEND

Antigens:

H-R/SE -SE antigens of H1-infected RK13 cells: top and bottom wells

H-F/SE -SE antigens of H1-infected F cells: centre well

Antisera:

RaHR-V -to H-R/V: purified H1 virions grown in RK13 cells: top trough

RaHF-V -to H-F/V: purified H1 virions grown in F cells: bottom trough

One viral antigen of H-R/SE and one viral antigen of H-F/SE, with similar electrophoretic mobilities, are identified by their reactions with antiviral sera RaHR-V and RaHF-V.

−
+
HR
SE
Ra
HR-V
HF
SE
Ra
HF-V
HR
SE

V. OPTIMAL CONCENTRATION OF ANTIGENS AND ANTISERA FOR IMMUNODIFFUSION ANALYSES

The effects of diluting SE antigens, and of inactivating (56°C for thirty minutes) or diluting homologous antisera were investigated by setting up immunodiffusion reactions in which different concentrations of SE antigens were allowed to react with varying dilutions of antisera. Plate A-II shows representative results, obtained when varying concentrations of H-F/SE were tested against homologous antiserum RaHF-S, and against dilutions of inactivated RaHF-S. A maximum of seven precipitin lines was produced when H-F/SE containing 8 mg protein per ml was allowed to react against undiluted RaHF-S or inactivated RaHF-S. Dilution of either RaHF-S or of H-F/SE resulted in the detection of fewer precipitin lines (figures a,b, and c).

On the basis of these results, and of similar results obtained when H-R/SE was tested with its homologous antiserum RaHR-S, all SE antigens were used at a standard concentration of 8 mg protein per ml, and all antisera were used undiluted and without inactivation for immunodiffusion analyses.

PLATE A-II

DETERMINATION OF OPTIMAL CONCENTRATIONS OF ANTIGENS AND ANTISERA FOR IMMUNODIFFUSION ANALYSES

LEGEND

Antigens:

H-F/SE - SE antigens of H1-infected F cells

H-F/SE 8 - diluted to contain 8 mg protein per ml
H-F/SE 4 - diluted to contain 4 mg protein per ml
H-F/SE 2 - diluted to contain 2 mg protein per ml

Antiserum:

RaHF-S - rabbit antiserum against H-F/SE

RaHF-S - uninactivated, undiluted
RaHF-S - inactivated at 56°C for thirty minutes
RaHF-S 2 - inactivated, diluted 1:2
RaHF-S 4 - inactivated, diluted 1:4
RaHF-S 8 - inactivated, diluted 1:8
RaHF-S16 - inactivated, diluted 1:16

Figure a) Reactions of H-F/SE containing 8 mg protein per ml. Seven antigens of H-F/SE react with undiluted RaHF-S, both before (RaHF-S un.) and after inactivation (RaHF-S). Only five of these antigens react with RaHF-S diluted 1:2, and only two antigens react with higher dilutions of antiserum.

Figure b) Reactions of H-F/SE containing 4 mg. protein per ml. Six antigens of H-F/SE react with undiluted antiserum. Only five antigens are detected by antiserum diluted 1:2, and only two by higher dilutions of antiserum.

Figure c) Reactions of H-F/SE containing 2 mg. protein per ml. Five faint precipitin lines are formed between H-F/SE and undiluted antiserum; fewer antigens react with higher dilutions of antiserum.

Ra
HF-S
Ra
HF-S
2
HF
SE-8
Ra
HF-S
4
Ra
HF-S
6
Ra
HF-S
8
a
Ra
HF-S
Ra
HF-S
2
HF
SE-4
Ra
HF-S
6
Ra
HF-S
8
b
Ra
HF-S
Ra
HF-S
2
HF
SE-2
Ra
HF-S
4
Ra
HF-S
6
Ra
HF-S
8
c