VESICULAR STOMATITIS VIRUS

VESICULAR STOMATITIS VIRUS: STUDIES OF VIRAL PROTEINS AND AUTOINTERFERENCE

BY

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The long-range aim of this thesis was to further the understanding of the mechanisms involved in virus interference. To this end, biochemical and immunological procedures were used to characterize and define the virus-specific proteins of VSV. Biological studies of homotypic and heterotypic interference were also carried out.

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ABBREVIATIONS

Ab	Antibody
Act D	Actinomycin D
Ag	Antigen
B	Infectious particle of VSV
BSA	Bovine Serum albumin
CF	Complement fixation
cpm	Counts per minute
DNA	Deoxyribonucleic acid
DOC	Deoxycholate
EDTA	Ethylenediaminetetraacetate
FCS	Fetal calf serum
HA	Hemagglutination
HR-LT	Heat resistant strain of Indiana VSV
IND-ST	Short T producing Indiana VSV
LT	Long T particle of Indiana VSV
MEM	Minimal essential medium
moi	Multiplicity of infection
NBCS	New born calf serum
NJ	New Jersey serotype of VSV
OD	Optical density
PBS	Phosphate buffered saline
PFU	Plaque forming unit

RBC	Red blood cell
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
rpm	Revolutions per minute
RSB	Reticulocyte standard buffer
S	Sedimentation coefficient
SDS	Sodium dodecyl sulphate
SDS-ME	Sodium dodecyl sulphate and 2-mercaptoethanol
ST	Short T particles of Indiana VSV
TCA	Trichloroacetic acid
ts	Temperature sensitive (mutants)
UV	Ultraviolet light
VBS	Veronal buffer saline
VP	Viral protein
VSV	Vesicular stomatitis virus

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INTRODUCTION

The discovery of viruses (Beijerinck, 1898; Loeffer and Frosch, 1898; Reed, 1902; Von Prowazek, 1907; Rous, 1911; d'Hérelle, 1917) and subsequent investigations into their structure and function have thrown light on intimate biological processes of great interest. Studies of the molecular events involved in viral replication and virus-induced cell alterations suggest not only a way of controlling viral diseases but also provide a model system for the study of genetic and regulatory mechanisms of cell function.

A virus, it is now realized, represents the simplest form of a self-replicating system and constitutes a separate category of living organism that has a mode of replication distinct from higher forms. They differ fundamentally from cells in that viruses contain only one type of nucleic acid, which may be either DNA or RNA, lack enzymes which function in energy metabolism, and have a distinct host-cell-dependent mechanism of multiplication.

Several hundreds of identified viruses of bacteria, plants and animals have been found to be capable of multiplication only in living cells of susceptible species. The information for genetic continuity of virus replication resides in the nucleic acid of the infecting virus. Unlike

cellular RNA, the nucleic acid of most RNA-containing viruses is self-replicating in a permissive host, the formation or participation of cellular or viral DNA is not required in their replication cycle. The important exceptions to this are the myxoviruses (Barry et al., 1962) and RNA tumor viruses (Temin, 1964; Bader, 1965; Fujinaga et al., 1970; Baltimore, 1970; Temin and Mizutani, 1970; Spiegelman et al., 1970).

It seems that, for most RNA viruses, the viral genetic information required for the production and assembly of virus specific macromolecules is contained within the virus particle itself. The translation of this genetic information into a complex series of biological events occurs only when viral nucleic acid gains access to cells in which it can function. With most viruses, this results in virtually complete domination of the cell by viral genetic material which can lead ultimately to death of the cells with concomitant release of viral progeny. However, infection of an appropriate cell strain with an oncogenic virus may lead not to death of the cell, nor of the production of viral progeny but rather result in neoplastic transformation of the cell.

In those cases where additional viruses are formed, the newly synthesized particles may be either released from or accumulate in the host cell. Most of these virus particles are capable of subsequent infection but some are defective

for their own replication.

The presence of non-self-replicating, defective particles is being recognized in an increasing number of mammalian virus systems (Uchida and Watanabe, 1969; Parks et al., 1968; Pons and Hirst, 1969; Huang and Baltimore, 1970; Mak, 1971; Cole et al., 1971). The role of the defective virus particle has been considered as a possible important determinant in the outcome of natural viral infection (Cooper and Bellett, 1959; Henle, 1963; Huang and Baltimore, 1970).

The term "defective virus" has been used to indicate non-replicating particles that are functionally deficient in some aspect of their replication. In a number of virus systems defective particles have been shown to be responsible for a specific type of viral interference (Huang and Baltimore, 1970).

Interference is measured by reduction in the yield of infectious particles produced in cells co-infected with both infectious and defective particles as compared to the yield from cells infected with infectious virus alone. Viral interference caused by defectives occurs in the absence of detectable interferon synthesis and appears to be dependent on a functional genome within the defective particles (Huang and Baltimore, 1970).

Vesicular Stomatitis Virus (VSV) is an ideal model system to study viral interference since the defective,

autointerfering particles produced by VSV are readily separable from the infectious virions.

1. The Natural History of Vesicular Stomatitis Virus

Vesicular Stomatitis Virus (VSV) is responsible for disease in a broad range of mammalian hosts (Theiler, 1901; Mohler, 1918; Hanson, 1952) and may be transmitted by arthropod vectors (Ferris et al., 1955). The disease in mammals is generally acute and self-limiting although the clinical symptoms in cattle and sheep are difficult to distinguish from the disease caused by foot and mouth disease virus. The symptoms of VSV infection were first reported by Hutcheon in 1898.

In 1925 cattle arriving in Richmond, Indiana from Kansas City showed symptoms of a disease which was later transmitted to horses. The infectious agent was first isolated by Cotton in 1926 and this strain of virus is now known as the Indiana serotype of VSV. In 1926, virus isolated from an outbreak in New Jersey was found to be immunologically distinguishable from the Indiana virus (Cotton, 1927) and this strain is known as the New Jersey serotype of VSV. The Indiana and New Jersey serotypes were found to possess at least one immunologically common antigen (Myers and Hanson, 1962).

In 1964, Jonkers et al. isolated Cocal virus in Trinidad Rodents (rise rats) and showed it to be serologically related to the Indiana serotype of VSV. The virions of Cocal

and VSV are morphologically indistinguishable (Ditchfield and Almeida, 1964; Bergold and Munz, 1967; Federer et al., 1967) and it was proposed on the basis of these findings that the Cocal virus is a subtype of Indiana serotype.

2. Growth in Tissue Culture and Mode of Development

VSV has very broad in vitro host range (Sellers, 1955; McClain and Hackett, 1958). Sellers (1955) demonstrated VSV multiplication in calf and pig kidney cultures as well as in chick embryo cells. McClain and Hackett (1958) extended the range of host cells to include monkey kidney, Earle's mouse L-cells and human HeLa cells. Human KB cells, VERO cells from African green monkey (cercopithecus), Chinese hamster ovary cells (CHO) and baby hamster kidney cells (BHK_{21}) are added to the above list by Mussgay and Weibel (1963), Schaffer et al. (1968), Stampfer et al. (1969) and Zee et al. (1970). In addition to this vertebrate tissue culture, Yang et al. (1969) have shown that the virus can also replicate in moth cell in tissue culture. The early stages of viral growth including adsorption and penetration have been examined by electron microscopy. Heine and Schnaitman (1969) claim that adsorption and penetration occur by an exact reversal of the extrusion process. The virion attaches to the cell surface usually at the flat end of the viral envelope and fuses with the cell membrane. The penetration occurs by release of the nucleoprotein into the cytoplasm.

Simpson et al. (1969) however, maintain that entry of VS virions is effected by viropexis, a process akin to phagocytosis. The true mechanism however is not clear yet.

VSV has been shown to mature at two different sites, the plasma membrane and the intracytoplasmic vacuolar membranes. Howatson and Whitmore (1962) described the plasma membranes as the principal site of virus maturation in L-cells, but also noted a few virus particles in intracytoplasmic vacuoles. Mussgay and Weibel (1963) demonstrated maturation of the virus at plasmic as well as intracytoplasmic vacuolar membranes in KB cells. Similar observations were made by Schulze and Liebermann (1966) in infected bovine kidney cells and David-West and Labzoffsky (1968) in chick fibroblasts. Hackett et al. (1968) and Zee et al. (1970) have demonstrated the site of viral maturation as observed by electron microscopy of thin section and concluded that the principle site of viral maturation was the intracytoplasmic vacuolar membranes for pig kidney cells and the cytoplasmic membranes for L-cells and VERO cells. Both types of membranes served as sites for HeLa and BHK_{21} cells. Therefore, it seems that the site of maturation of VSV is a host dependent phenomenon.

Cytoplasmic aggregates of nucleoproteins are not readily detected in section of infected cells though analysis of polyribosome extracts of the virus infected L-cells showed that unenveloped nucleoprotein strands are present in appreciable quantities (Schincariol and Howatson, 1970; Petric and Prevec, 1970)

3. Alteration of Host Cell Metabolism upon VSV Infection

The ability of a virus to affect host cell function is an important aspect of the infection process. The viral infection may result in the shutoff of host cell macromolecular synthesis or it can stimulate and induce the synthesis of certain host cell specific products such as interferon.

Infection by VSV produces a rapid and pronounced decrease in the rate of cellular RNA synthesis. Huang and Wagner (1965) reported that the cytotoxicity of VSV for Krebs-2 carcinoma cells of mice seemed to be related to the rapid inhibition of cellular RNA synthesis. As expected, cellular DNA and protein synthesis are also compromised as are other cellular functions including the capacity to synthesize interferon (Wagner and Huang, 1966). The cytoplasmic membrane subsequently becomes permeable to the dye eosin, and the cells undergo marked metabolic and morphologic changes. Other investigators have shown that VSV is toxic for cells infected at high multiplicities (Cooper and Bellett, 1959; Turco, 1959) even after infectivity has been inactivated by ultraviolet irradiation (Cantell et al., 1962; Wagner et al., 1963). In 1966, Huang and Wagner showed that VSV was unable to stimulate interferon synthesis, but could inhibit the production of interferon induced in Krebs-2 cells previously infected with Newcastle disease virus. They pointed out that VSV acted much like Actinomycin D

(Reich et al., 1961) in its effect on RNA and interferon synthesis and concluded that the inhibition of host RNA synthesis was responsible for the failure of VSV to induce interferon and its ability to inhibit Newcastle disease virus induced interferon synthesis. While infection of L-cells with wild type VSV at high multiplicities does not induce the production of interferon, infection with low multiplicities of a smaller plaque mutant results in the synthesis of large amounts of interferon (Wertz and Younger, 1970).

4. Morphology and Fine Structure of VSV

Infectious virus particles: The main structural features of the VS virion have been established by electron microscopy of thin section of infected cells and negatively stained viral preparations (Chow et al., 1954; Bradish et al., 1956; Howatson and Whitmore, 1962; Simpson and Hauser, 1966; McCombs et al., 1966; Bradish and Kirkham, 1966; Nakai and Howatson, 1968). There is general agreement that the infectious particles are bullet shaped and accordingly are referred to as B particles. The B particle is cylindrical with one hemispherical and one planar end, and consists of an internal helical structure which appears as a series of transverse striations surrounded by a membranous envelope with fine outer filamentous surface projections (Fig. 1

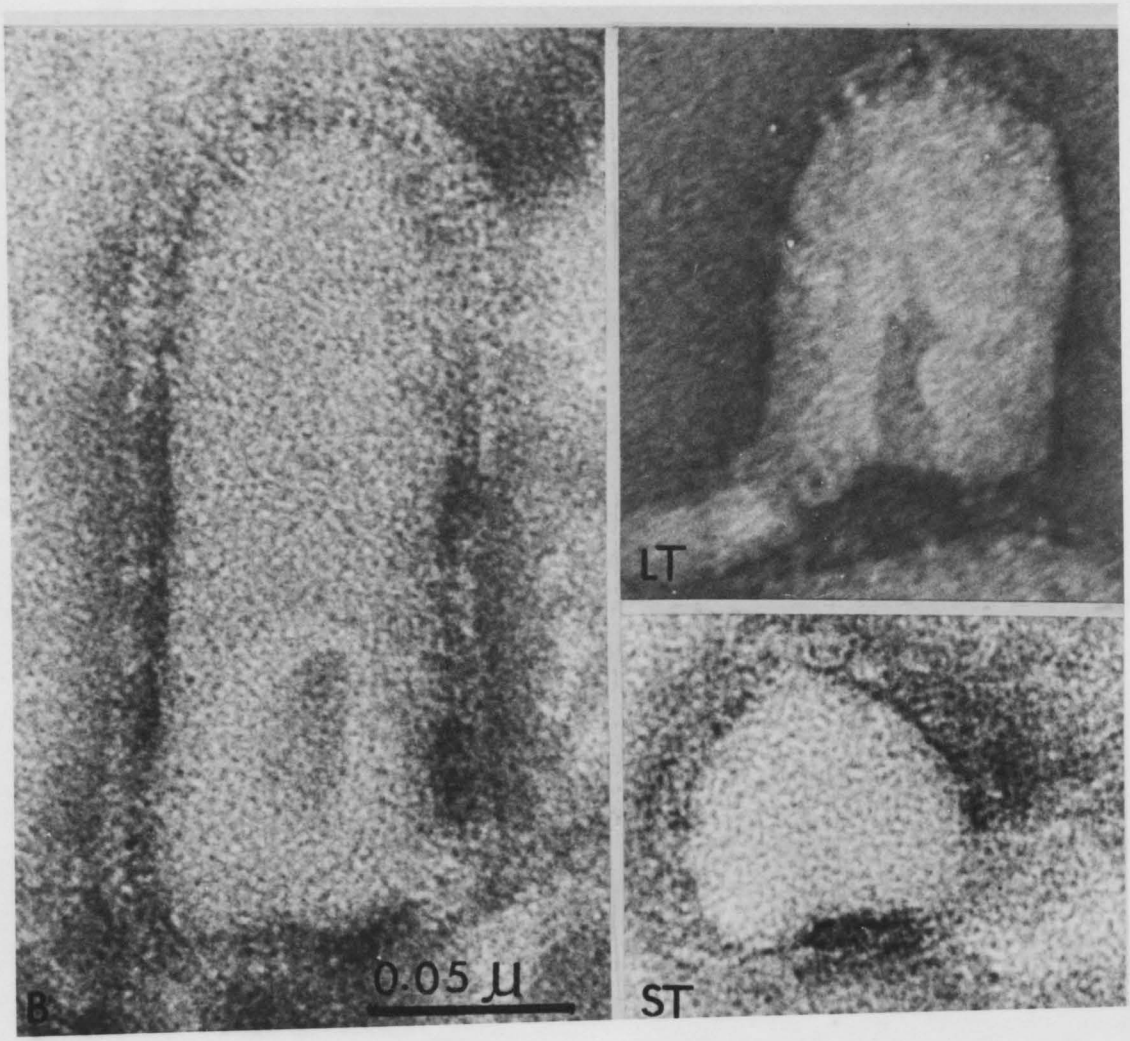


Fig. 1. Electron micrographs of VSV.
The preparation was negatively stained with 2% phosphotungstic acid and examined on a Philips EM300. Mag. X 500,000.

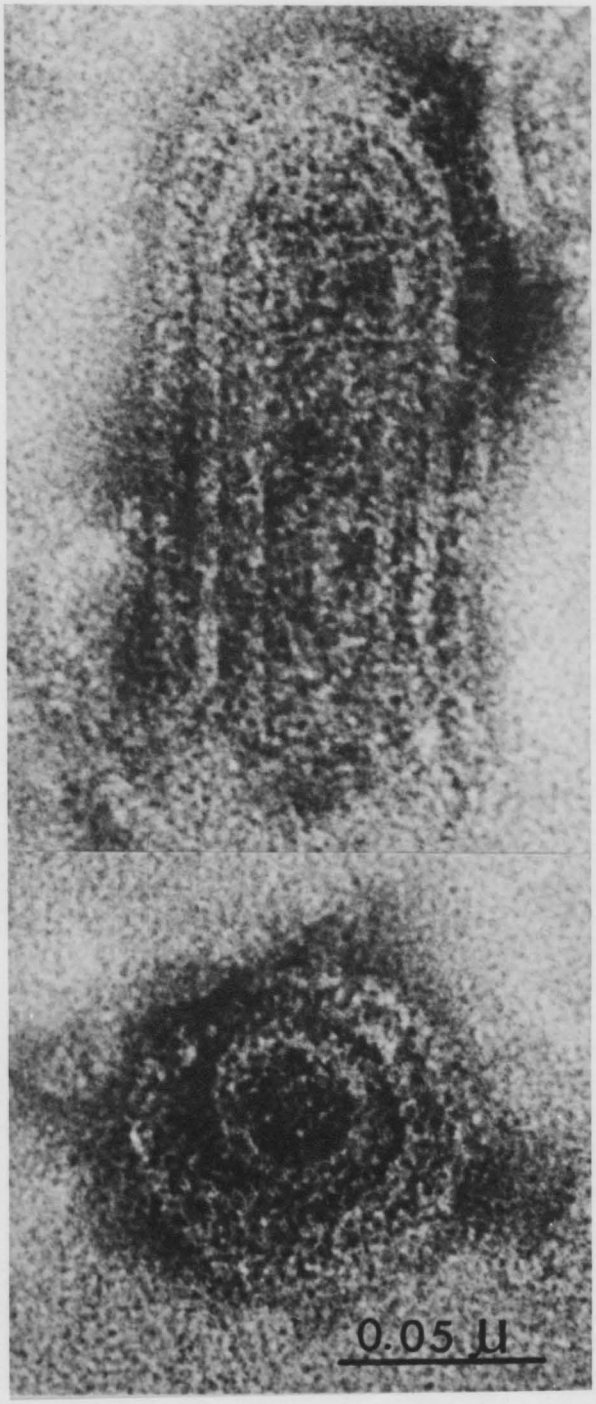


Fig. 2. The internal structure (top) and cross sectional view (bottom) of VSV. Mag. X 500,000.

and Fig. 2). There is a central axial channel which is often partially penetrated by the stain in negatively stained preparations. The estimated dimensions of B particle are approximately 175 mμ in length, 65 mμ in diameter; with surface projections some 10 mμ long attached to the envelope at approximately 4.5 mμ intervals. There appear to be no significant morphological differences between virions of different serotypes (Bergold and Munz, 1967).

Several different models of the virion have been proposed (Simpson and Hauser, 1966; Bradish and Kirkham, 1966; Bergold and Munz, 1967; Nakai and Howatson, 1968). Nakai and Howatson suggest that the nucleoprotein is in the form of a single helix of about 30 turns capped by four or five turns of diminishing diameter at the round end. The helix consists of approximately 1000 subunits, each of dimensions approximately 90Å x 30Å x 30Å, the long axis of the subunits being radially oriented. The continuity of the helix is maintained by a single-stranded RNA molecule.

Defective virus particles: Repeated undiluted high multiplicity passage of VSV in cell culture results in reduced yields of infectious virus and the concomitant appearance of a shorter, truncated, defective particle (Cooper and Bellett, 1959; Bellett and Cooper, 1959). The equivalence of this truncated or T particle to the "transmissible interfering component" of VSV preparations described previously by Cooper

and Bellett (1959) and Bellett and Cooper (1959) was demonstrated by Huang and Wagner (1966). Electron microscopic examination of the truncated particles revealed that they were morphologically indistinguishable from the B particles in all respects except overall length (Fig. 1). Hackett (1964) demonstrated that T particles produced by virus of New Jersey serotype were 65 mμ in diameter and 85 mμ long while Huang et al. (1966) observed that T particles produced by virus of the Indiana serotype were 65 mμ in both diameter and length. Petric and Prevec (1970) observed that T particles produced by a heat resistant (HR) strain of Indiana VSV were 100 mμ in length (designated LT) contrasting to the 65 mμ particles (designated ST) produced by the original parental Indiana VSV. The infectious particles of the HR and parental strain are identical in length.

Both LT and ST contain helically wound ribonucleoprotein structures morphologically similar to those derived from B particles but shorter in length by approximately the same ratio as that of the length of the virus particles (Nakai and Howatson, 1968; Petric and Prevec, 1970).

5. Homologous Viral Interference

As has been stated previously, the defective T particles, either ST or LT are known to interfere with the production of infectious B particles. The origin of the T particles and their role in interfering with the replication

of VSV are being investigated in several different laboratories.

Working with New Jersey serotype, Hackett (1964) found the defective particles present only in suspensions which had been produced by multicyclic growth of either dilute or undiluted passage virus.

Using the Indiana serotype, Huang et al. (1966) found that undiluted passage of virus in chick embryo cell monolayers produced a population of virus particles which contained considerable numbers of T particle whereas diluted passage virus contained very little of this component.

Huang and Wagner (1966) showed that ST particles of the Indiana serotype, which are capable of interfering with the production of infectious virus of the Indiana serotype (homotypic interference), are much less effective in interfering with the production of infectious virus of the New Jersey serotype (heterotypic interference).

The following information has been obtained concerning the mechanism of homotypic interference itself; a) Simultaneous infection of cells by B and T particles lowers the yield of infectious virus produced (Cooper and Bellett, 1959). b) Interference does not occur at the level of virus adsorption or penetration since addition of T particles to infected cells anytime within the virus latent period (1.5 hours) can reduce virus yield (Huang and Wagner, 1966). c) Addition of T particles to infected cells after

the latent period has no effect on virus yield, showing that an early viral function is inhibited (Huang and Wagner, 1966). d) Interference by T particle is not mediated by interferon since no interferon can be demonstrated in infected Krebs-2 carcinoma cells and interference can occur in the presence of Actinomycin D (Huang and Wagner, 1966). e) UV irradiation of T particles abolishes their interfering activity, suggesting that functional T particle RNA is required for interference (Huang et al., 1966). This hypothesis is strengthened by the observation of Sreevalsan (1970) that RNA isolated from ST particles is capable of interfering with B particle replication in chick embryo culture.

6. Viral Antigens

In 1956 Bradish et al. described four antigenic components of the VSV system separable on the basis of sedimentation coefficient. The infectious, 625s component was shown to consist of rods of diameter 175 x 69 mμ. A second 330s component was in the form of granules or spheres of diameter 65 mμ and has little or no infectivity. About 35% of the total complement fixing (CF) activity was associated with 625s and 330s components. It is now clear that the 625s and 330s components can be identified with B and ST particles, respectively.

Two smaller antigens with approximate sedimentation coefficients of 20s and 6s are also produced by VSV infection and these components contain the remaining 65% of the CF activity.

These antigens have been studied immunologically by Brown et al. (1966, 1966a, 1967) however, relatively little is known of their functional origin or significance.

Brown and his collaborators studied the effect of Tween-ether, trypsin and phospholipase C on the structure and antigenic properties of VSV. The Tween-ether treatment reduced infectivity of the preparations by about 10,000 fold but enhanced the CF activity and immunogenicity for guinea pigs.

Treatment with trypsin for five minutes was found to reduce infectivity 100,000 fold and to destroy the immunizing activity of the virus preparation. The CF activity with antiserum to the whole virus was also removed by trypsin. In contrast, treatment with phospholipase C reduced the infectivity only slightly and the immunizing activity and CF activity with antiserum to virus remained unaffected. However, the CF activity with antiserum directed against host cell components was greatly reduced by phospholipase C treatment (Cartwright et al., 1969). These investigators concluded that the surface projections consist of virus protein and are responsible for the immunizing activity and virus specific CF activity of the virus, and

that a phospholipid component derived from the host cell is located in the regions between the projections. Other evidence indicating that a cellular component is present in the envelope of the virus was obtained by Cartwright and Pearce (1968).

The presence of a viral hemagglutinin in VSV capable of agglutinating goose erythrocytes at a low temperature and appropriate pH was described by Arstila et al. (1969). The hemagglutinating activity was shown to be associated with the whole virus particle but the particular component responsible has yet to be determined.

7. Viral Genome and Virus-specific RNAs in Infected Cells

Evidence that VSV is an RNA containing virus was first presented by Prevec and Whitmore (1963) and indirect evidence by Chamsy and Cooper (1963). This RNA was first characterized by Huang and Wagner (1966) who showed that the sedimentation coefficients of RNAs from B and ST particles averaged 43s and 23s, respectively. Using the emperical formula developed in 1963 by Spirin ($M = 1.55\ S_{20,W}^{2.1}$) where M is molecular weight, and $S_{20,W}$ is the sedimentation coefficient, the above authors have estimated that the molecular weights of B and ST particle RNAs to be 4.0×10^6 and 1.3×10^6 respectively. The RNA extracted from both B and ST particles were readily solubilized by treatment with RNase indicating that the molecules were single-stranded.

Similar results were obtained independently by Brown et al. (1967a). the molecular weight of B particle RNA was estimated as 3.0×10^6. Nakai and Howatson (1968) measured the length of unwound nucleoprotein strands derived from disintegrated VSV particles. The average length of the ribonucleoprotein strand is 3.5 μ for B particles and 1.1 μ for ST particles yielding RNA molecular weights of 3.5×10^6 and 1.1×10^6 respectively assuming that the linear density of single-stranded nucleic acid is 100 daltons per Å. Petric and Prevec (1970) have estimated the molecular weight of LT particle genome as 1.7×10^6 from its sedimentation coefficient of 30s - 32s.

Several species of RNA have been found in virus infected cells during replication of VSV. Schaffer et al. (1968) observed RNase-sensitive RNA at 6, 15, 31 and 38s in VERO cells examined 17 hours post-infection. Newman and Brown (1969) using BHK_{21} cells labelled with radioactive RNA precursors up to 5 hours postinfection found five peaks of RNA at <4, 12, 20, 28 and 38s. The peaks contained varying amounts of RNase resistant RNA. Stampfer et al. (1969) reported two groups of RNA species in VSV infected CHO cells; group one included RNAs made when cells were producing mainly B particles and included single-stranded components at 13, 28, 40s and partially double-stranded 23s to 35s RNA; group two consisted of RNA species made when the majority of the particles produced were T particles and included single-

stranded 6 s and 19s, partially double-stranded 15s and 19s and completely double-stranded 13s species.

Schincariol and Howatson (1970) found that VSV infected L-cells synthesize at least six single-stranded viral specific RNA components designated 13, 15, 21, 26, 31 and 38s. The 26 and 38s components have the same sedimentation coefficient as the RNA from LT and B particles. The 13 and 15s RNAs are the predominant species synthesized during the infection cycle. RNA species sedimenting at 21 and 31s are always found in small amounts. Two classes of double-stranded RNA sedimenting at 13 to 15s and 19 to 20s are found in small quantities and it is postulated that these are replicative-forms of LT and B respectively. Partially RNase-resistant RNA sedimenting heterogeneously from 13 to 50s are also found and it is postulated that these represent replicative-intermediates.

Recently, Huang and Stampfer (1970) have examined RNA extracted from the polyribosomes region of sucrose density gradients of VSV infected cell cytoplasmic extracts. Only the 12s RNA species was implicated as messenger-RNA based on the fact that ethylenediaminetetraacetic acid (EDTA) treatment of the cytoplasmic extracts released this RNA species from the polyribosomes.

8. Particular Area of Concern

In order to understand the formation of defective virus particles and the phenomenon of viral interference, one must have information regarding the nature of viral antigens. Accordingly, studies were initiated to identify and characterize the viral specific proteins released from VSV infected cells, the viral specific protein synthesis in infected cells and the effect of added defective particles on infectious particle replication.

A complete biological and biochemical study of the VSV system should provide clues to many basic mechanisms of RNA replication and regulation of protein and RNA synthesis in mammalain systems. Furthermore, an understanding of the mechanisms of homologous interference, may well point the way to develop controls for a number of different viral infections.

MATERIALS AND METHODS

MATERIALS

1. Source of Cells

A continuous-passage line of Earle's L-cell (Earle, 1943) designated L-60 was obtained from Dr. A. F. Howatson, Department of Medical Biophysics, University of Toronto. Growing cultures of human KB cells were obtained from Dr. S. Mak, Department of Biology, McMaster University, while human FL cells and primary chick embryo cells were kindly provided by Dr. D. R. E. MacLeod, Connaught Medical Research Laboratories, University of Toronto.

2. Source of Viruses

Two strains of the Indiana serotype of VSV were obtained from Dr. A. F. Howatson. Of these, one strain, here designated IND-ST, was identical to the virus used in previous work (Nakai and Howatson, 1968). The second strain, designated HR-LT, was selected from IND-ST stock by Dr. T. Nakai for its resistance to high temperature (43°C). The selection procedure involved repeated heating at 43°C for 3 hours followed by passage of the survivors

in L cells at 37°C. The final selected strain was purified by plaque picking (see text) on L cell monolayers.

Virus of the New Jersey (NJ) serotype was obtained from Dr. J. Campbell, School of Hygiene, University of Toronto.

3. Growth Medium

Joklik modified minimal essential medium (MEM) was purchased in powder form from the Grand Island Biological Company (Gibco). The powder was dissolved in glass distilled water to the prescribed concentration and sterilized by filtration through a Millipore filter having a pore diameter of 0.22 μ.

MEM containing one-twentieth the normal amino acid concentration was formulated using Earle's salts solution and concentrates of MEM-Vitamins, MEM-Antibiotics and MEM-Amino acids purchased from Gibco.

Fetal calf serum (FCS) or New Born Calf Serum (NBCS), purchased from Gibco, was added to MEM in appropriate concentrations prior to use.

4. Chemicals and Solutions

All chemicals used were of analytical reagent grade.

(a) Phosphate Buffer Saline (PBS) (Dulbecco and Vogt 1954)

NaCl	8	gm
KCl	2	gm
$NaHPO_4$	1.15	gm
KH_2PO_4	2	gm

made up to 1 liter with glass distilled water

pH 7.5

(b) Reticulocyte Standard Buffer (RSB°) (Prevec, 1965)

NaCl	0.58	gm
$MgCl_2.6H_2O$	0.30	gm
Tris	1.21	gm

made up to 1 liter with glass distilled water and adjusted to pH 7.5 with concentrated HCl.

(c) RSB^+

Similar to RSB° but containing 10 fold higher concentrations of NaCl and $MgCl_2.6H_2O$.

(d) Veronal Buffer Saline (VBS)

NaCl	83	gm
Na.5-5 diethylbarbiturate	10.19	gm

dissolved in 1 liter of glass distilled water

1 N HCl	34.58	ml
1 M $MgCl_2$ + 0.3 M $CaCl_2$ solution	5	ml

made up to 2 liters with glass distilled water and stored at 4°C. For use, the stock solution is diluted 1:5 in glass distilled water to give a final pH of 7.3.

(e) Virus Disrupting Buffer (Summer et al., 1965)

Urea	7.5 gm
SDS	5.0 gm
Acetic Acid (glacial)	50.0 gm
KH_2PO_4	0.39 gm
Na_2HPO_4	1.36 gm

made up to 100 ml with glass distilled water.

(f) Sodium dodecylsulphate - Mercaptoethanol solution (SDS-ME)

SDS	1 gm
2-mercaptoethanol (100%)	1 ml

made up to 100 ml with glass distilled water.

(g) Alkylating Buffer (Reuckert, 1965)

Urea	48 gm
SDS	1 gm
EDTA	0.19 gm
2-mercaptoethanol (100%)	0.25 ml
phenol red	0.005 gm

made up to 100 ml with 0.5 M Tris buffer pH 8.6.

(h) Phosphate Buffer (Polyacrylamide gel (PAG) running buffer) (Summer et al., 1965)

Na_2HPO_4	13.6 gm
KH_2PO_4	3.87 gm
SDS	1 gm

made up to 1 liter with glass distilled water

pH 7.2

(i) Dialysis Buffer (Summer et al., 1965)

PAG running buffer	100	ml
Urea	30	gm
SDS	0.9	gm
2-mercaptoethanol (100%)	1	ml

made up to 1 liter with glass distilled water.

(j) Acrylamide Gel Solutions

Solution A

- For 5% polyacrylamide gel with normal cross-linker

Acrylamide (Eastman Organic Chemicals)	10	gm
NN-Methylenebisacrylamide	0.2	gm

made up to 100 ml of PAG running buffer (h)

- For 7.5% polyacrylamide gel with various cross-linker

Acrylamide	15	gm
NN-Methylene bisacrylamide		
High cross-linker	0.4	gm
Normal cross-linker	0.3	gm
Low cross-linker	0.2	gm

made up to 100 ml of PAG running buffer (h).

Solution B

N.N.N',N Methyltetraethylenediamine	0.1	ml
PAG running buffer (h)	100	ml

Solution C

Ammonium persulphate	0.56	gm
PAG running buffer (h)	100	ml

(k) Coomassive Blue Solution

Coomassive blue	0.25	gm
Methanol	25	ml
Acetic acid (glacial)	10	ml

made up to 100 ml with glass distilled water.

(l) Destaining Solution

Methanol	25	ml
Acetic acid (glacial)	10	ml

made up to 100 ml with glass distilled water.

(m) Scintillation Fluids

- Bray's solution (Bray, 1960)

2,5 diphenyloxazole (PPO)	12	gm
1,4-bis 2 (5 phenyloxaxolyl) benzene (POPOP)	0.6	gm
Methanol	300	ml
Naphthalene	180	gm
Ethylene glycol	60	ml

made up to 3 liters with para-1,4 dioxane.

- Toluene based fluid

PPO	4	gm
POPOP	0.3	gm
Toluene	1	liter

5. Biological Compounds and Radiochemicals

Complete Freund's adjuvant was purchased from Difco, while guinea pig complement and hemolysin were purchased from the Microbiological Associates Inc., Bethesda. Sheep red blood cells (RBC) were purchased from the Connaught Medical Research Laboratories, Toronto.

Act D was a generous gift from Dr. W. D. Dorian of Merck, Sharp and Dohme, Montreal. Acti-dione (cycloheximide) was purchased from the Upjohn Company, Kalamazoo, Michigan.

The proteins used for reference markers were obtained from Nutritional Biochemical Corporation: γ-globulin; from Sigma: aldolase, BSA, cytochrome c (horse heart), hemoglobin (bovine heart) and urease; from Worthington: alkaline phosphotase, catalase, trypsin, pepsin, chymotrypsinogen and pepsinogen. Myosin samples were gifts from Drs. Susan Lowey (Harvard Medical School) and W. F. Harrington (The Johns Hopkins University) phosphorylase b samples were gifts from Drs. N. Madsen (University of Alberta) and P. Cohen (University of Washington).

Amino acid mixture labelled with either ^{14}C or ^{3}H were purchased from the New England Nuclear Corporation. Approximate specific activities were 1.54 mc/1 mgm and 25 mc/1 mgm for ^{14}C and ^{3}H amino acid mixture respectively.

D-glucosamine labelled with ^{14}C was purchased from the New England Nuclear Corporation. The specific activity was 32.5 μc/1 mgm in 0.5 ml of 70% ethanol.

METHODS

6. Tissue Culture

L-cells and KB cells were grown in suspension culture in MEM supplemented with 5% FCS or NBCS. The suspension cultures were kept in a tightly stoppered erlenmeyer flask containing a sterile teflon coated magnetic bar and incubated in a 37°C water bath equipped with magnetic stirrers.

The cell concentration was measured using a Levy ultraplane hemocytometer and the culture was kept in constant exponential growth phase by daily two-fold dilution.

FL cells and primary chick embryo cells were grown in 32 oz Brockway bottles incubated at 37°C for 3 to 4 days until full cell monolayer had formed.

7. Assay for Plaque Forming units (PFU) and Methods of Plaque Purification

To determine the plaque-forming titer of a virus production, 0.1 ml of appropriately diluted virus was placed directly on a full L-cell monolayer, preformed in

Falcon petri dishes (60 x 15 mm).

The virus was allowed to adsorb for 30 to 45 minutes at 37°C in a atmosphere of 5% CO_2 in air and 100% humidity. The infected cell sheet was then overlaid with 5 ml of solution containing MEM growth medium, 5% FCS and 0.9% of Noble agar (Difco). Plaques could be counted without staining after incubation for 20 to 24 hours at 37°C in a water saturated, 5% CO_2 atmosphere.

To purify by "plaque picking" a sterile Pasteur pipette was inserted through the agar over a visible plaque. The area of the plaque was scraped with the pipette tip and a small amount of fluid along with the agar plug was removed and dispersed by pipetting in one ml of growth medium. Retitration of this material and a second plaque pick followed. Generally viruses were carried through three successive plaque purifications to ensure some degree of genetic homogeneity of the initial virus stocks.

8. Preparation of Stock Virus

Stock virus was obtained by three successive passages of plaque purified viruses, each passage at a multiplicity of infection (moi) of 0.01 PFU per cell. For virus passage, exponentially growing cells at 4×10^5 cells/ml were centrifuged at 400 x g for 10 minutes. The cell pellet was suspended to 10^7 cells/ml

in 10 ml of MEM containing 2% FCS and appropriately diluted virus. The virus was allowed to adsorb in this volume by incubation for 45 minutes at 37°C in suspension culture. After this time MEM containing 2% FCS was added to produce a final cell concentration of 0.5×10^6 to 10^6 cells/ml. Incubation was continued in suspension culture at 37°C for 8 to 14 hours, after which time cells and cellular debris were removed by centrifugation at 400 x g for 10 minutes and the supernatant stock virus suspension aliquoted and stored at -60°C until further use. The virus titre in the stock preparations ranged from 0.5×10^9 to 10^9 PFU/ml. Under these conditions, the stock virus contained mainly B particles as determined by sucrose gradient centrifugation.

In order to obtain T particle containing virus stocks, the above stock virus was passaged repeatedly at multiplicities of approximately 1000 PFU/cell. After four or five such passages the resultant virus stock contained approximately equivalent numbers of B and T particles.

9. Preparation and Analysis of Sucrose Gradient

Linear gradients of 5 to 30% or 15 to 30% sucrose dissolved in PBS or RSB were prepared in 5, 30 or 38 ml volumes, as required by using a Buchler gradient maker. The gradient tubes were then placed in Beckman SW50, SW25.1, or SW 27 rotor buckets and chilled to 4°C. No more than

1.5 ml of material was layered on the 30 or 38 ml gradients, and no more than 0.2 ml on the 5 ml gradients. These were centrifuged at an appropriate speed and 5°C for the desired time in an L2-65B ultracentrifuge. The gradient fractions were usually collected in successive 1 ml or 0.2 ml with continuous monitoring of the optical density (254 mμ or 280 mμ) of the effluent using an Isco UV analyzer and fraction collector. This instrument collects a gradient beginning at the top or lighter portion.

For radioactive analysis a portion of each fraction was made 5% (w/v) in ice cold TCA and the resultant precipitate collected with suction on a nitrocellulose filter (0.45 μ Sartorius). The acid precipitate was washed with 5 ml of ice cold 5% TCA using suction filtration and the filters dried in an oven at 100°C. The filters were then immersed in 5 ml of toluene-based scintillation fluid and radioactivity measured on Beckman LS-250, LS-233 or LS-100 liquid scintillation counters.

On some occasions, approximately 0.02 ml of the gradient fraction was placed directly into 5 ml of Bray's scintillation fluid and the radioactivity was measured.

10. Preparation of Radioactively Labelled Viral Components

Some 5×10^8 L-cells were collected by centrifugation and resuspended in 25 ml of MEM with 2% FCS containing enough virus to give a moi of 50 PFU/cell. Adsorption was

carried out in this volume in suspension culture at 37°C for 45 minutes and the culture was then diluted to 400 ml with prewarmed MEM containing 2% FCS. The time of dilution is taken as time zero of infection. After 3 hours of incubation at 37°C, the infected cells were pelleted by centrifugation at 400 x g for 10 minutes and suspended in 200 ml of prewarmed medium which was identical to MEM except that it contained only one-twentieth the normal amino acid concentration and was supplemented with 2% FCS. After 30 minutes of incubation at 37°C, 70 µc of a ^{14}C-amino acid mixture was added to the culture and incubation was continued for a further 5 to 6 hours. At this time, the culture was harvested by centrifugation at 500 x g for 15 minutes. The cell pellet resulting from this centrifugation was discarded, and only the supernatant fluid was carried through subsequent fractionations.

The cell-free virus fluid was centrifuged at 81,000 x g for 75 minutes at 5°C in a Beckman model L2-65B ultracentrifuge. The pellet of this centrifugation, which contains almost all of the viral infectivity of the culture, was resuspended in PBS and was purified by centrifugation on a 5-30% linear sucrose gradient.

The supernatant fraction of the centrifugation of the cell-free virus fluid contains the 20S and 6S viral antigens. These were precipitated from solution by the dropwise addition of 1.5 volumes of a saturated solution of

ammonium sulphate as described by Brown et al. (1966). Precipitation was allowed to occur at 4°C for a further 60 minutes. The precipitate was collected by centrifugation at 16,000 x g for 20 minutes and washed once with 50% saturated ammonium sulphate solution by resuspension and further centrifugation. The precipitate was then dissolved in 1 ml of PBS and dialyzed overnight against 1000 volumes of PBS. After dialysis, the antigens were fractionated by centrifugation on a 5-30% linear sucrose gradient.

11. Immunological Procedures

A. Preparation of Antisera: Antisera were produced in a rabbit using the following schedules and routes of injection: Primary injections of antigen with complete Freund's adjuvant in 0.5 ml volumes were given intramuscularly and intraperitoneally. Three subsequent weekly injections beginning 14 days after the primary injections were given without adjuvant, 0.5 ml intraperitoneal and 0.1 ml intravenous. One week after the last injection the rabbit was bled by exsanguination through a carotid catheter and the serum collected after clotting. All sera were then heated at 56°C for 30 minutes and stored at -60°C until further use.

For use as antigen, whole virus was grown in human KB cells, purified by sucrose gradient centrifugation and suspended to a concentration of 10^9 PFU/ml. Viral core

and coat antigens were prepared as described in the results and a total of one mgm of protein used for the inoculations.

B. Complement Fixation Test (CF): Complement fixing titer was determined by using microtiter technique described by Sever (1962). Box titrations were employed to determine optimal complement and hemolysin titers. The 50% hemolysis end-point was determined visually. Antibody was standardized by box titration with a standard VSV lysate used as antigen and with one optimal unit of hemolysin and three units of complement. For the experimental CF determinations, four units of antibody were used.

C. Neutralization Test: The neutralization titer of antiserum was determined by incubating standard amounts of virus with appropriate antiserum dilutions for 30 minutes at 37°C. The residual infectivity was determined by PFU assay procedures.

D. Serum Blocking Power Test: Serum blocking power was measured by mixing dilutions of antigen with a known titer of virus-neutralizing antiserum. After incubation at 37°C for 6 hours, a standard amount of virus was added to each tube. Incubation was continued at 37°C for 30 minutes, and the residual infectivity in each tube was then determined by PFU assay.

C. Immune Precipitation Test: Immune precipitates of viral antigens were prepared by mixing antigen with an equal volume of antiserum and incubating the mixtures at 4°C for

2 days. The precipitates were collected by centrifugation at 7,000 x g for 30 minutes and washed twice with 95% ethanol containing 0.1 M ammonium acetate. The radioactivity in the precipitated antigen was then determined.

12. Extraction of Proteins from Infected Cells

Infected and radioactively labelled cells prepared as previously described were collected by centrifugation at 600 x g for 20 minutes. The cell pellet containing some 3×10^7 cells was then solubilized by resuspending it in five volumes of SDS-ME solution. The material was treated with ultrasonics for 30 seconds at the lowest intensity setting of a Biosonik III (Bronwill Scientific). The solubilized viscid sample was placed in an ice-bath and ten volumes of ice-cold 95% ethanol added. After one hour the precipitated protein was collected by centrifugation at 600 x g for 20 minutes. The precipitate was dissolved in approximately five volumes of alkylating buffer. The sample was allowed to stand at room temperature for one hour and then dialyzed overnight against 1000 volumes of dialysis buffer.

13. Extraction of Cytoplasm from Infected Cells

At 5 hours post-infection, infected cells were harvested from the medium by centrifugation at 800 x g for 5 minutes at 4°C in a PR-2 centrifuge and chilled on ice. The cell pellet was washed three times by successive

resuspension and centrifugation in ice-cold PBS. Following the third washing the cell pellet was resuspended in ice-cold RSB° to a cell concentration of 10^8 cells/ml and the cells allowed to swell for 10 minutes. The swelled cells were then disrupted by 10-12 strokes of a tight fitting Dounce homogenizer and the homogenate centrifuged at 7000 rpm for 5 minutes in a Sorvall RC-2B refrigerated centrifuge. This procedure yielded a pellet and milky supernatant. The supernatant was further analyzed on a 15-30% sucrose gradient made in RSB° or RSB^+.

14. Polyacrylamide Gel Analysis

The solubilized and dialyzed protein samples were subjected to electrophoresis on polyacrylamide gel. Polyacrylamide gel columns (bed volume of 0.9 cm in diameter by 17 cm in length) were performed in plastic tubes (27 cm long with inner diameter 0.9 cm purchased from Buchler Instruments). The tubes were sealed at one end with dialysis bag held by a small elastic band. The acrylamide gel solutions A, B and C were mixed in a ratio of 4:3:1 respectively, urea added to a final concentration of 0.5 M and the resultant solution poured into the vertical plastic tubes. Approximately 0.5 ml of water was carefully layered on top of the gel mixture and the mixture allowed 45 minutes to polymerize at room temperature. The water on top of the polymerized gel was then removed and no more than 0.3 ml of protein

sample dissolved in dialysis buffer containing approximately 20% sucrose was applied on the gel. The polyacrylamide gel running buffer was carefully layered on the sample to fill the plastic tube. The tube containing polyacrylamide gel and protein sample was then placed vertically in a Buchler-gel electrophoresis stand and running buffer added to the reservoir chambers.

The running buffer was maintained at approximately 25°C to prevent precipitation of SDS. An initial field strength of 1 volt/cm of gel was applied for 30 minutes, and the voltage was increased to 4 volts/cm of gel. The constant current was approximately 25 mA at approximately 70 volts/17 cm gel.

The gel, after 20 to 22 hours electrophoresis and being freed from the walls of the plastic tube with slight water pressure, was sliced manually with a razor blade into discs approximately 0.12 cm in height. Each disc was placed directly into a scintillation vial containing either 5 ml of Bray's solution or 10 ml of toluene based scintillation fluid containing 25% methanol. The samples were counted in a Beckman model LS-250 scintillation counter. When dual label (^{3}H and ^{14}C) was employed in one gel, the gel slices were first depolymerized by incubating at 50°C for 4-5 hours in 0.1 ml hydrogen peroxide per slice and then counted after addition of 5 ml of Bray's scintillation fluid.

In order to recover proteins from the gel the appropriate gel slices were individually incubated for 3 days at 4°C in 0.5 ml of 1 mM unbuffered ammonium bicarbonate. Recovery was about 70% using this procedure.

For non-radioactive samples, the electrophoresed gels were stained one hour with Coomassive blue solution at 37°C and destained at 65°C with two changes of the destaining solution in 30 minute intervals. Destaining continued in a solution containing 10% acetic acid at room temperature.

15. Sedimentation Velocity Analysis

Ethanol precipitated protein samples were treated with alkylating buffer and dialyzed three days in 5000 volumes of 0.01 M phosphate buffer containing 0.05 M urea, 0.05 % SDS and 0.05% 2-mercaptoethanol with three changes of dialysis buffer.

Sedimentation velocity analyses were carried out in a Beckman model E analytical ultracentrifuge using Schlieren optics. Approximately 2 mgm protein in one ml of above dialysis buffer was placed in a standard sector-shaped cell and centrifuged at 56,000 rpm at 20°C. At least 3 pictures were taken at 8 or 16 minute intervals for each protein sample after the sample had moved from the meniscus.

The distance from the meniscus to the Schlieren peak marking the boundary of the sample has been measured by

Nikon Profile Projector model 6c.

The viscosity and density of the buffer solution was measured at 20°C ± 0.1 and $S_{20.w}$ was calculated by Svedberg's equation (Svedberg and Pedersen, 1940)

$$S_{20.w} = \frac{S_t \left(\frac{\eta_t}{\eta_{20.w}}\right)(1 - V_{20}\rho_{20.w})}{(1 - V_t \rho_t)}$$

where the subscript w denotes water, v partial specific volumn of protein, η viscosity of buffer, ρ density of buffer and S_t was determined in the buffer solution at t°C.

16. Amino Acid Analysis

Amino acids were analyzed on request by Dr. W. W. Chan, Department of Biochemistry, McMaster University.

Samples were hydrolyzed in 6 N HCl at 110°C for 24, 48 and 72 hours in an evacuated test tube. After hydrolysis, the samples were analyzed for amino acid content using the Beckman amino acid analyzer.

17. Electron microscopy

A suspension of the sample to be studied was spotted on a formvar-coated carbonized grid of 2-300 mesh and allowed 1 minute to adhere. The bulk of the suspension was removed with a piece of filter paper. The sample was then stained negatively with 2% phosphotungstic acid at pH 6.8 adjusted

with KOH and after 1 minute the stain was blotted off with a piece of filter paper. When the RNP was being examined, the material on the grid was negatively stained by adding one drop of 2% uranyl acetate to the sample, waiting for 1 minute, then blotting off the excess stain and drying. The grids were observed and photographed on Philips 300 electron microscope.

RESULTS

1. Demonstration of Homologous Viral Interference and of Defective Particle Production

Repeated undiluted high multiplicity passage of VSV in cell culture results in an inhibition of infectious virus particle production and the concomitant appearance of shorter, truncated, defective particles. This phenomenon is illustrated by the experiment described in Fig. 3.

For this experiment virus was plaque purified five consecutive times in order to obtain virus stocks relatively free of T particles. The resultant stock virus was then passed at a multiplicity of infection (moi) of 100 PFU/cell six consecutive times. The yield of infectious virus at each passage was measured by plaque assay after 24 hours of infection and the results were plotted. Although there is an initial increase in the titre of infectious virus produced at early passages, it is evident that continuous high moi passage results in a rapid decrease of infectivity by the fifth passage.

Since this figure shows only the yield of infectious virus as measured by PFU, a subsequent experiment was performed in order to determine the effect of continued high moi passage on total particle production.

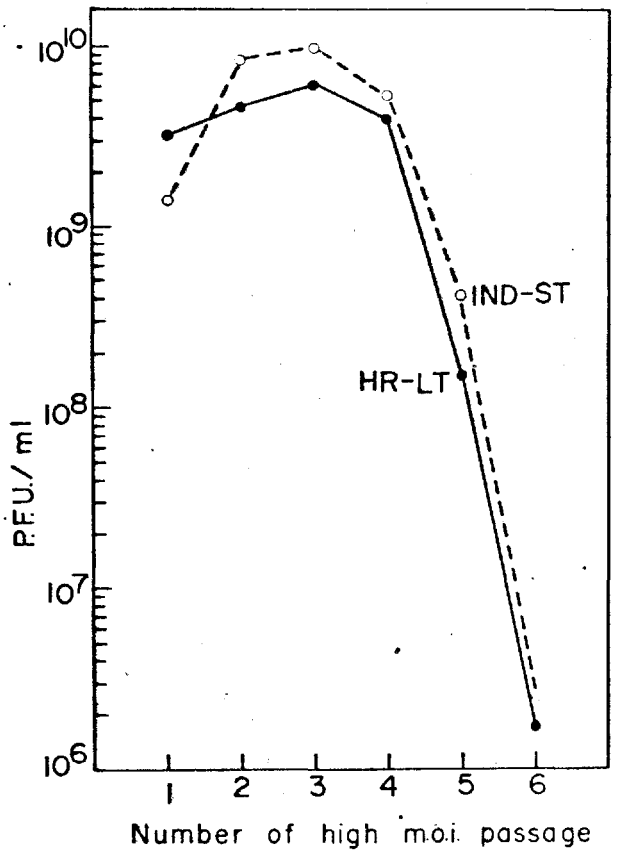


Fig. 3. Effect on infectious virus yield of continuous high multiplicity passages.
Suspension culture of some 10^8 cells were infected with 10^{10} plaque forming units (PFU) of plaque purified B particles of either IND-ST or HR-LT strain. After 30 minutes absorption at 37°C the infected cells were resuspended in MEM to 10^6 cells/ml and incubated at 37°C in suspension culture for 24 hours.
The yield of infectious virus was measured by plaque assay.

B and T particles can be separated on sucrose gradients and quantitated by optical density measurement. Accordingly, the lysate of cultures from each high moi passage was harvested and after partial purification by differential centrifugation, the particle yields were analyzed on sucrose gradients as shown in Fig. 4.

Before gradients presented in Fig. 4 can be fully understood it is necessary to describe the nature of the various components contributing to the optical density profiles. The predominant optical density peak evident in the 1st through 4th passage which is located at fractions 22 and 23, is due to infectious B particles. An electron microphotograph of material taken from a comparable region of the sucrose gradient is labelled "B" in Fig. 5, which also shows that material taken from positions of sucrose gradients labelled "ST" and "LT" in Fig. 4 do indeed contain "short T" and "long T" particles respectively. The profile in Fig. 4 indicates that ultraviolet absorbing material is present in a fraction that sediments faster than B particles (fraction 27 approximately) and in a fraction near the top of the gradient (fractions 3 to 7). On electron microscopic examination, the material sedimenting ahead of B particles appeared to consist of small clumps of the same particles. The reason for its rather homogenous sedimentation profile is unknown. Electron microscopic examination of material from the top

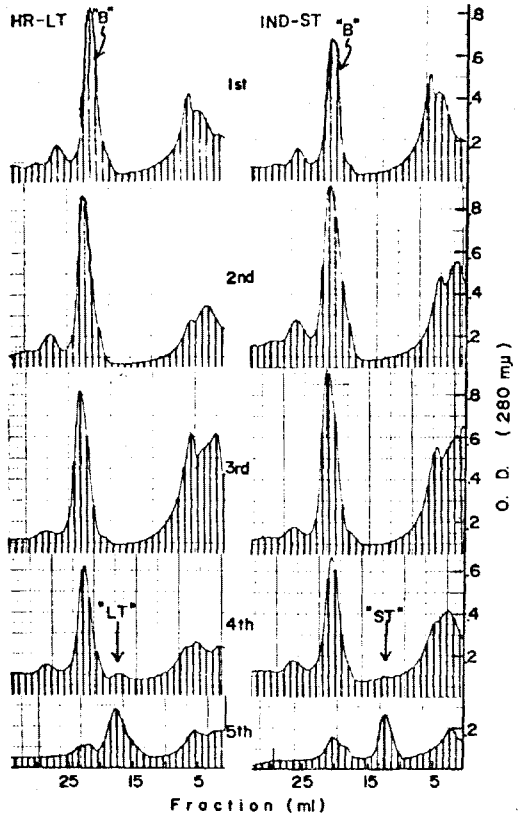


Fig. 4. Effect of high multiplicity passages on the total particle yield of cells infected either with HR-LT or IND-ST strain.
Samples of lysate from each passage illustrated in Fig. 3 used. After removal of cells and debris the virus particles were pelleted from the supernatant by centrifugation at 59,000 x g for 120 minutes, resuspended in 1 ml of PBS and layered on a linear 5-30% sucrose gradient in PBS. After centrifugation for 81,000 x g at 4°C for 35 minutes the gradients were collected and the effluent continuously monitored for optical density (280 mμ) using an Isco gradient fractionator. The strains used, fractions of particles, and the number of each successive passage are indicated.

of the sucrose gradients revealed structures which resembled the ribonucleoprotein component of virions (labelled RNP in Fig. 5). Petric and Prevec (1970) have reported that ribonucleoproteins of both B or T particles accumulate within infected cells. When virus is harvested late in infection the nucleoprotein is found in the extracellular fluid possibly as a result of cellular disruption. On occasion a shoulder of UV-absorbing material is found on the light or trailing side of the B particles. Electron microphotographs of this material (labelled EFB in Fig. 5) suggest it consists of B particles with envelopes either greatly damaged or completely absent.

Returning to the experiment presented in Fig. 4 we will consider the effect on B and T particle production only of continuous undiluted virus passage. It can be seen that up to the third passage, large amounts of B particles continue to be produced and there is no indication of any T particle production. By the fourth passage, T particles become detectable; on the fifth passage the number of B particles produced is greatly decreased while the number of T particles is increased. The coincidence of the decrease in B particles with T particle increase is in agreement with the hypothesis that interference is mediated by T particles.

In order to more clearly define interference, the effect of PFU and particle production of mixed infection with purified T particles and plaque purified B particles was next examined.

Fig. 5. Electron microphotographs of fractions taken from Fig. 4.

ST: short T particles from 5th passage of IND-ST strain (fractions 12-13).

LT: long T particles from 5th passage of HR-LT strain (fractions 17-18).

B: B particles from 1st passage of IND-ST strain (fractions 22-23).

EFB: envelope-free-B particles from 2nd passage of IND-ST strain (fractions 19-20).

RNP: ribonucleoproteins from 1st passage of IND-ST strain (fractions 4-6).

All the virus particles were negatively stained with 2% phosphotungstic acid while RNP was negatively stained with 2% uranyl acetate.

Mag. X 150,000.

RNP
EFB
ST
0.1μ
LT
B

2. Interference by Purified LT and ST Particles

Long T particles were prepared from lysates of 5th high moi passage stock of HR-LT strain while short T particles were obtained from lysates of 5th high moi passage stock of IND-ST strain of VSV. Both long T and short T particles were separately purified by at least three consecutive sucrose gradient purifications. The final residual infective titre in T particle preparations is less than 10^4 PFU/ml.

The yield of infectious virus produced by cells infected with either strain of Indiana B particles together with either "long T" or "short T" particles is dependent on the multiplicity of defective particles added. At high dilution of T particles, where the interference effect is least, there is a near linear relationship between the log PFU and the dilution of the respective T particle stock (Fig. 6). The initial slope of the interference curve can be used as a measure of the interfering activity of a T particle preparation (Cooper and Bellett, 1959).

Fig. 6 indicates that both "short T" and "long T" particles are capable of homotypic interference with the HR-LT strain of VSV. The result, as it is plotted, does not show a real comparison of the interfering capacity of the two particles since the numbers of interfering particles per dilution are not equal. A real comparison can be obtained by mixing equal quantities of the "long T" and "short T" preparations, counting the ratio of the two

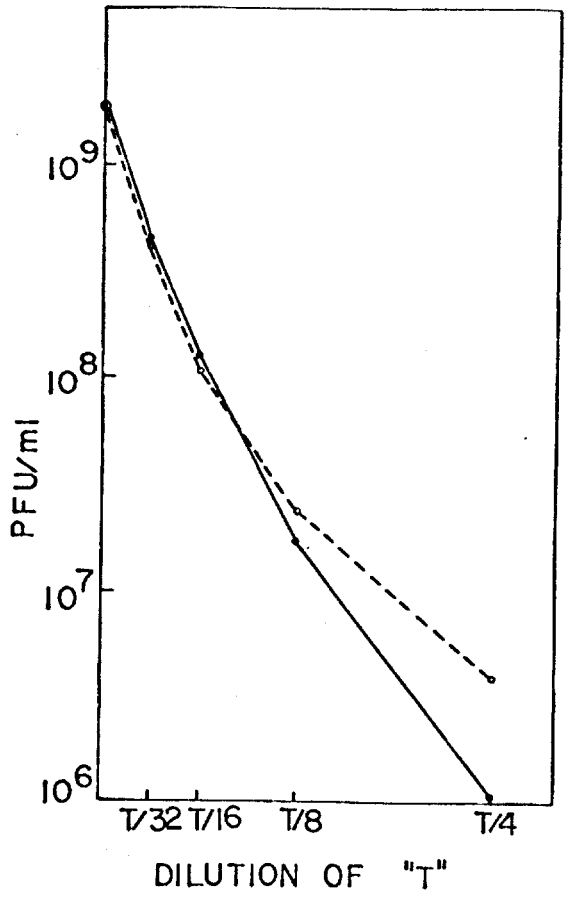


Fig. 6. Homotypic interference by "long T" and "short T" particles.
L-cell monolayers, 4 x 10^6 cells in sterile 60 x 15 mm. Falcon plastic petri dishes, were infected with a total of 4 x 10^6 PFU in 0.1 ml of B particles of the HR-LT strain. A similar volume of either growth medium or appropriately diluted "long T" (●) or "short T" (o) particle stock was also added to each plate. After absorption at 37°C for 30 minutes in an atmosphere of 5% CO_2 and 100% humidity, 5 ml of MEM growth medium supplemented with 5% fetal calf serum was added and the plates incubated a further 18 hours. At this time the yield of PFU/ml in each cell-free lysate was determined by plaque assay.

particles by electron microscopic examination, and then correcting the dilution factor of one type of T particle by this empirical ratio.

Using this normalizing factor the interfering activities of both "long T" and "short T" particle preparations with B particles of three virus strains were compared (Fig. 7). The solid line in this figure and the associated vertical bars represents the slopes of the linear portion of interference curves obtained for all the following combinations: "Short T" particles with B particles of the homotypic HR-LT and IND-ST strains, "long T" particles with both homotypic strains and "long T" particles with B particles of the New Jersey serotype. The upper dotted line represents the interference result obtained with "short T" particles of the IND-ST strain and B particles of the New Jersey serotype.

Thus the interfering activities of both "long T" and "short T" particles are identical when tested against homotypic B particles. In contrast, while "long T" particles are as effective in heterotypic as in homotypic interference, the "short T" particles are incapable of heterotypic interference.

3. Virus Particle Production During Interference

The interference assay determines the effect of interference on infectious B particle production only.

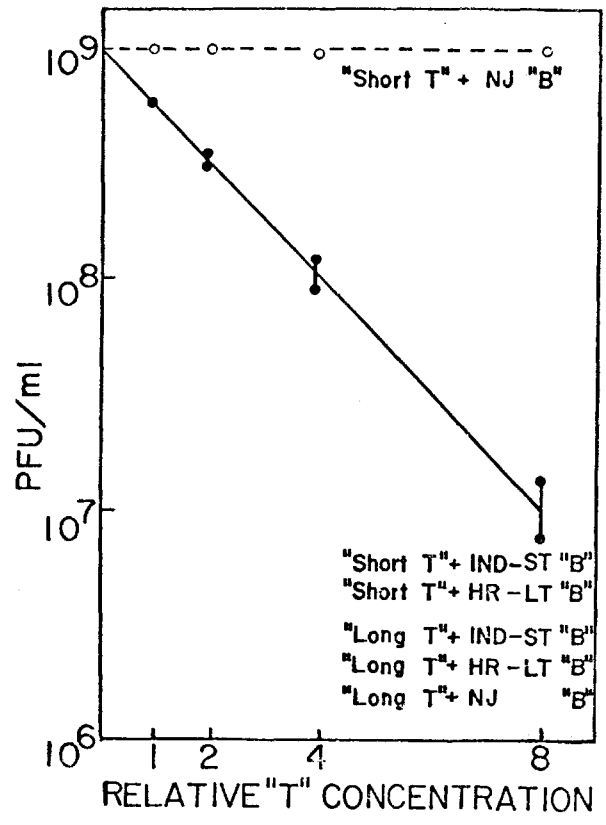


Fig. 7. Homotypic and heterotypic interference by "long T" and "short T" particles.
Interference curves for various combinations of T and B particles were obtained by procedures outlined in Fig. 6. At least three different experiments were done for each particle combination using two distinct "long T" and "short T" preparations. For each T particle preparation the relative number of "long T" to "short T" in the same volume of preparation was determined by direct microscope counting. The dilution factor of one particle type was corrected by this ratio and all results normalized to a control particle yield of 10^9 PFU/ml. The result as plotted above allows a direct comparison of all results. The solid line and associated bars shows the range of results obtained for the following five combinations: "short T" with homotypic HR-LT and with IND-ST B particles, "long T" with heterotypic New Jersey B particles. The broken line (o) represents the results of "short T" with heterotypic New Jersey B particles.

Consequently, experiments were done to examine the entire extracellular particle yield under each condition of interference by quantitation with optical density measurement after sucrose gradient analysis.

Fig. 8 shows the particle yield from cells infected with B particles of the HR-LT strain and coinfected with either long T or short T. With either T particle it can be seen that the greater the input of defective particles the fewer B particles are produced, as expected from infectivity assays. At low levels of interference the total mass of particle production does not change significantly since the loss in B particles is compensated by an increase in corresponding T particle production. At high levels of interference however, the actual particle production is in fact decreased.

The reduction of PFU by interference in the homotypic system therefore appears to be divided into two processes. At low levels of interference there is a progressive shift from synthesis of B particles to synthesis of T particles while at high levels of interference there appears in addition to be a real suppression of all types of particle synthesis.

Another very important conclusion can be drawn from the results presented in Fig. 8. Although the HR-LT strain of VSV does not under normal undiluted passage conditions, give rise to "short T" particles, it can, when superinfected

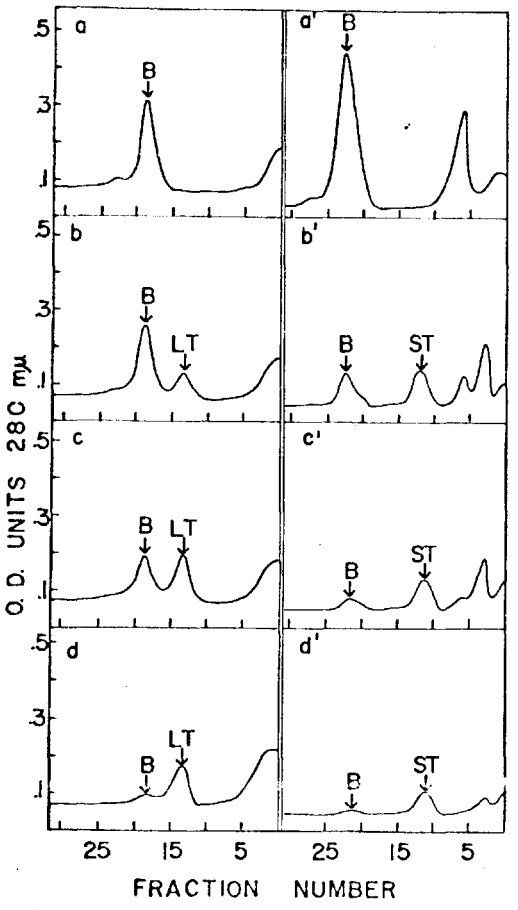


Fig. 8. Effect of "long T" and "short T" particles on the total particle yield of cells infected with HR-LT strain. Some 10^8 cells were infected with 1 PFU/cell of HR-LT B particle stock with or without the addition of appropriately diluted T particles. After absorption for 30 minutes at 37°C the cells were resuspended to 10^6 cells/ml and incubated in suspension culture for 10 hours (a,b,c,d) or 24 hours (a',b',c',d'). Cells and debris were removed from the lysate by centrifugation at 2,000 x g for 10 minutes. Virus particles were pelleted from the supernatant by centrifugation at 59,000 x g for 120 minutes, resuspended in 1 ml of PBS and layered on a linear 5 to 30% sucrose gradient in PBS. After centrifugation at 81,000 x g and 4°C for 35 minutes the gradients were collected and the effluent continuously monitored for optical density (280 mμ) using an Isco density gradient fractionator. a, a'-yield from cells infected with B particles alone. b, c, d-yield from cells infected with B particles plus two-fold increasing numbers of "long T" particles respectively. b', c', d'-yield from cells infected with B particles and increasing numbers of "short T" particles. The direction of sedimentation was from right to left.

with "short T" particles, lead to their replication.

This suggests that the defective particle is acting as a template for its own synthesis, presumably at the RNA replication level.

Identical results to those just described are obtained when the above experiments are repeated using the homotypic IND-ST strain of B particles as infectious virus with either long T or short T particles (Fig. 9).

Simultaneous addition of both long T and short T particles to cells infected with either strain of Indiana B particles results in the production of both types of T particles (Fig. 10) showing that replication of one T particle did not have a large selective advantage over replication of the other T particle.

Having determined that replication of both T particles accompanies interference in mixed infection, it was of great interest to determine the course of particle production in heterotypic interference. In this later situation we have seen that interference is mediated only by "long T" particles (cf Section 2).

When "long T" particles are used in heterotypic interference (Fig. 11b,c,d), there is a progressive reduction in the number of B particles produced as the input number of "long T" particles increase. In contrast to homotypic interference however, there is only minimal indication of T particle production. If "long T" particles

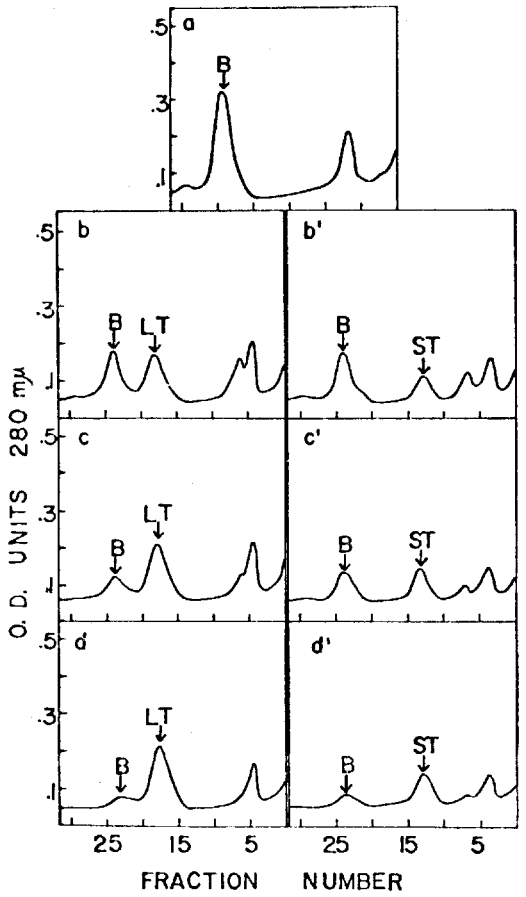


Fig. 9. Effect of long T and short T particles on the total particle yield of cells infected with IND-ST strain. Except for the difference in the strain of infectious virus, experimental conditions were identical to those in Fig. 8a', b', c', d'.

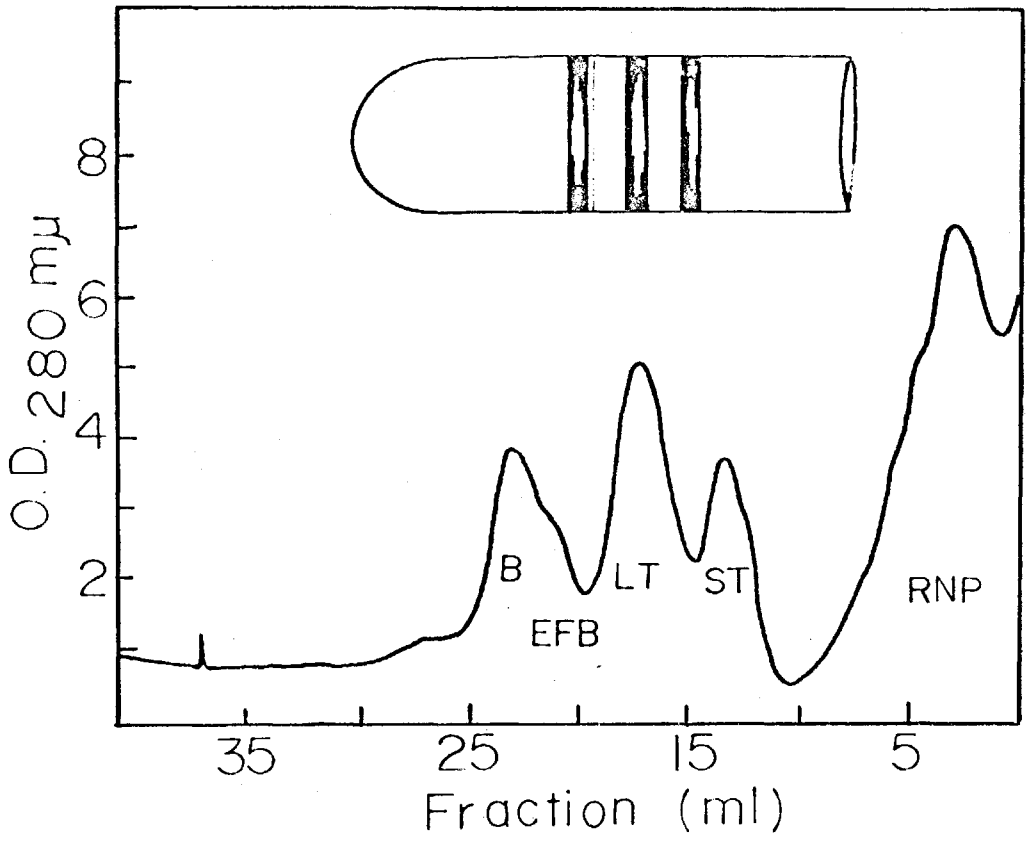


Fig. 10. Effect of simultaneously added both long T and short T particles to cells infected with HR-LT strain. Approximately 10^8 cells were infected with 1 PFU/cell of HR-LT B particle stock with the addition of appropriately diluted LT and ST particles. After 30 minutes absorption at 37°C the infected cells were resuspended to 10^6 cells/ml and incubated in suspension culture for 24 hours. Cells and debris were removed from the lysate by centrifugation at 2,000 x g for 10 minutes. Virus particles were pelleted from the supernatant by centrifugation at 59,000 x g for 120 minutes, resuspended in 1 ml of PBS and layered on a linear 5-30% sucrose gradient made in PBS. After centrifugation at 81,000 x g at 4°C for 35 minutes the gradients were collected and the effluent continuously monitored for optical density (280 mμ) using an Isco density gradient fractionator. Each peak represents as has labelled. The inset of this figure represents the light scattering bands visibly observed along the gradient prior to fractionation.

are indeed produced in this infection, their number is not related to the input of "long T" particles.

When "short T" particles are used in heterotypic combination with infectious B particles of the New Jersey serotypes it can be seen from Fig. 11b',c',d' that the yield of B particles remains unchanged. This is as expected from the lack of interference as determined by the yield of PFU (cf Fig. 7). At the same time it should be noted that no concomitant synthesis of "short T" particles occurs under these conditions. Thus the New Jersey serotype appears incapable of acting as a helper for synthesis of "short T" particles of the Indiana serotype. This result is not due to exclusion of "short T" particles from the cell by New Jersey B particles since preinfection with "short T" particles gives the same result.

As a control it should be pointed out that the results of two gradient analyses have been deleted since infection with "long T" or "short T" particles in the absence of added B particles at the highest concentrations used in this study, yielded neither light scattering nor optical density bands.

In order to gain a better understanding of the mechanism of viral interference and formation of defective virus it becomes necessary at this stage to obtain information regarding the nature of the virus genome and the proteins coded for by the virus. Accordingly studies

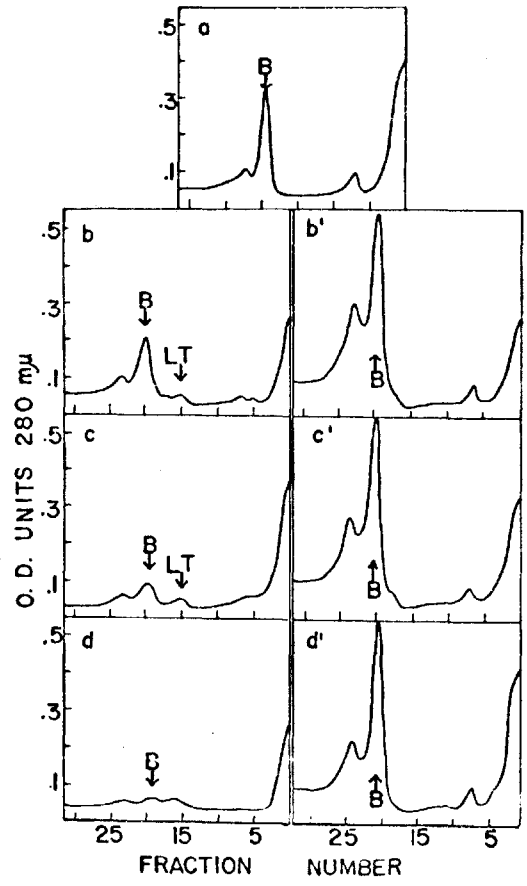


Fig. 11. Effect of "long T" and "short T" particles on the particle yield of heterotypic New Jersey B particle infected cells.
The procedures are identical to those described in Fig. 9 except that B particles of the New Jersey serotype were used. All cultures were harvested at 24 hours. The virus particles of b, c, d, were pelleted by centrifugation 81,000 x g for 70 minutes and somewhat greater particle recoveries were obtained than in a, b, c, d. a - B particles alone. b, c, d - B particles plus increasing numbers of "long T" particles. b', c', d' - B particles plus increasing numbers of "short T" particles. The direction of sedimentation is from right to left.

were initiated to identify and characterize the virus specific proteins contained within the virion and any related antigens produced during VSV infection.

4. Increase of Infectivity (PFU) and Complement Fixing (CF) Antigen Following Infection

Prior to the examination of individual virus proteins it was necessary to determine the time of appearance of virus components - infectious virus as measured by PFU and virus antigens as determined by CF activity - following infection under conditions comparable to those employed in subsequent experiments.

Fig. 12 illustrates the time course of viral infectivity and CF activity in an L-cell culture infected with the HR-LT strain of Indiana serotype. At various times post-infection, samples of 1 ml volume were removed and 0.5 ml was immediately frozen, then thawed and assayed for total PFU and CF activity. The other portion was centrifuged at 400 x g for 10 minutes to remove cells, and the supernatant fraction was assayed for free CF and PFU activity.

The infectious virus in the total culture begins to increase at 1 hour post-infection and increases exponentially until 6 hours post-infection (Fig. 12). Very little infectious virus remains cell-associated, supporting the hypothesis that virus matures and gains its infectivity on passage through the L-cell membrane (Howatson and Whitmore, 1962).

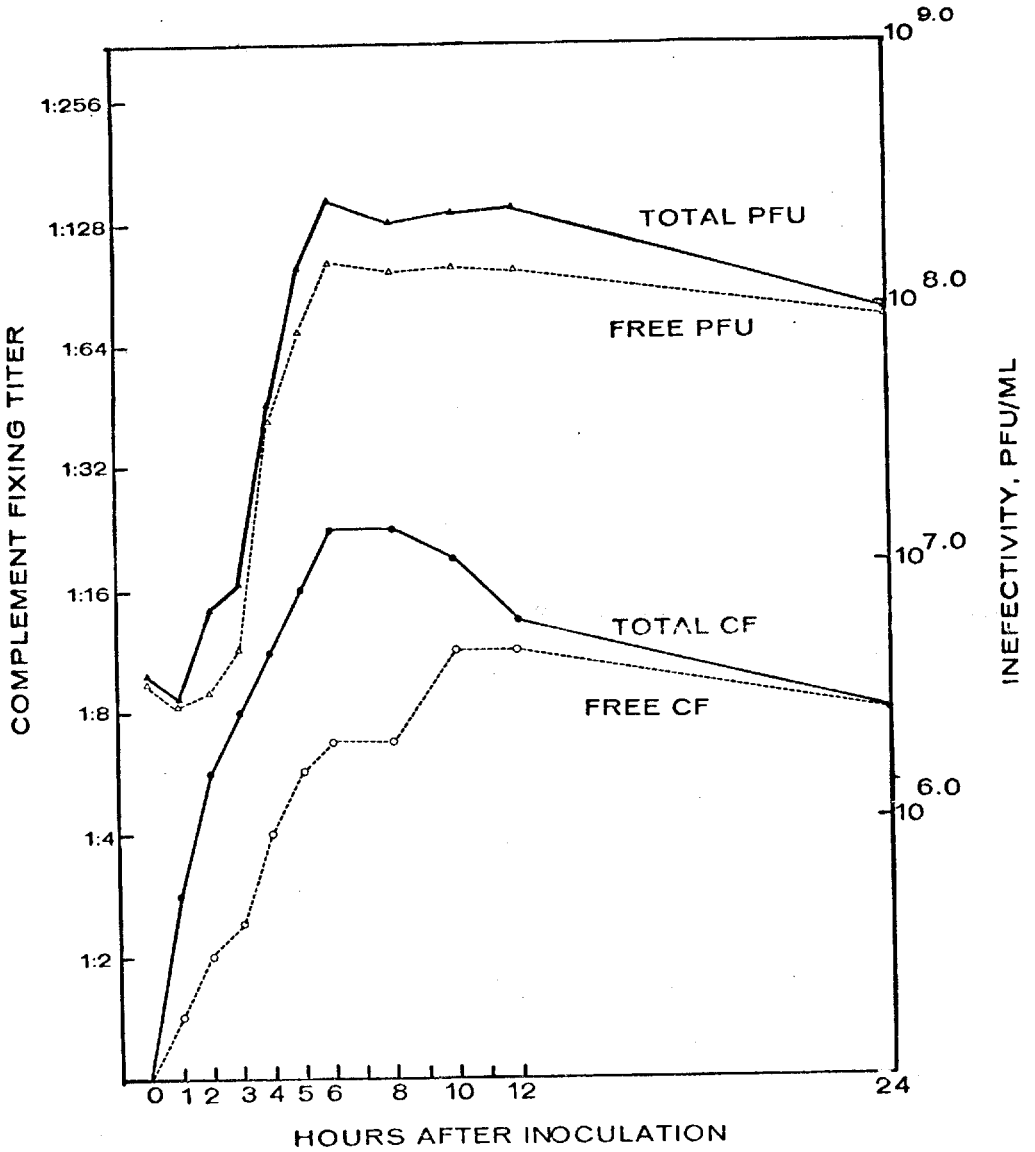


Fig. 12. Kinetics of PFU and CF activity.
A culture of L-cells was infected with VSV at moi of 50 as described in text. Time zero is taken after a 45 minute adsorption period. At indicated times, post-infection, samples were removed and PFU and CF determined in both total sample after freezing and thawing (total CF and PFU), and in cell-free sample (free CF and PFU).

The total CF activity increases from 1 to 6 hours post-infection. In the early stages a large proportion of the CF antigen is cell-associated. By 10 hours post-infection however, most of the CF activity is found in the extracellular fraction.

To ensure that the increase of CF activity with time after infection is in fact due to continued synthesis of protein antigen, the experiment shown in Fig. 13 was performed. The procedure was basically the same as that described above, except that the infected culture was split at 2 hours post-infection into two equal portions, and cycloheximide was added to one portion. Only the total PFU and CF activity were determined.

The addition of cycloheximide, an inhibitor of protein synthesis (Watanabe et al., 1967), caused an immediate cessation in the increase of both PFU and CF activity.

It is therefore concluded that CF activity in the infected culture results from de novo synthesis of virus-specific proteins.

5. Purification of ^{14}C-Labelled VSV Components

Since incorporated radioactivity was to serve as marker for viral proteins in subsequent analysis it was necessary to ensure that there was a direct correspondence between radioactive components and the CF or PFU activity

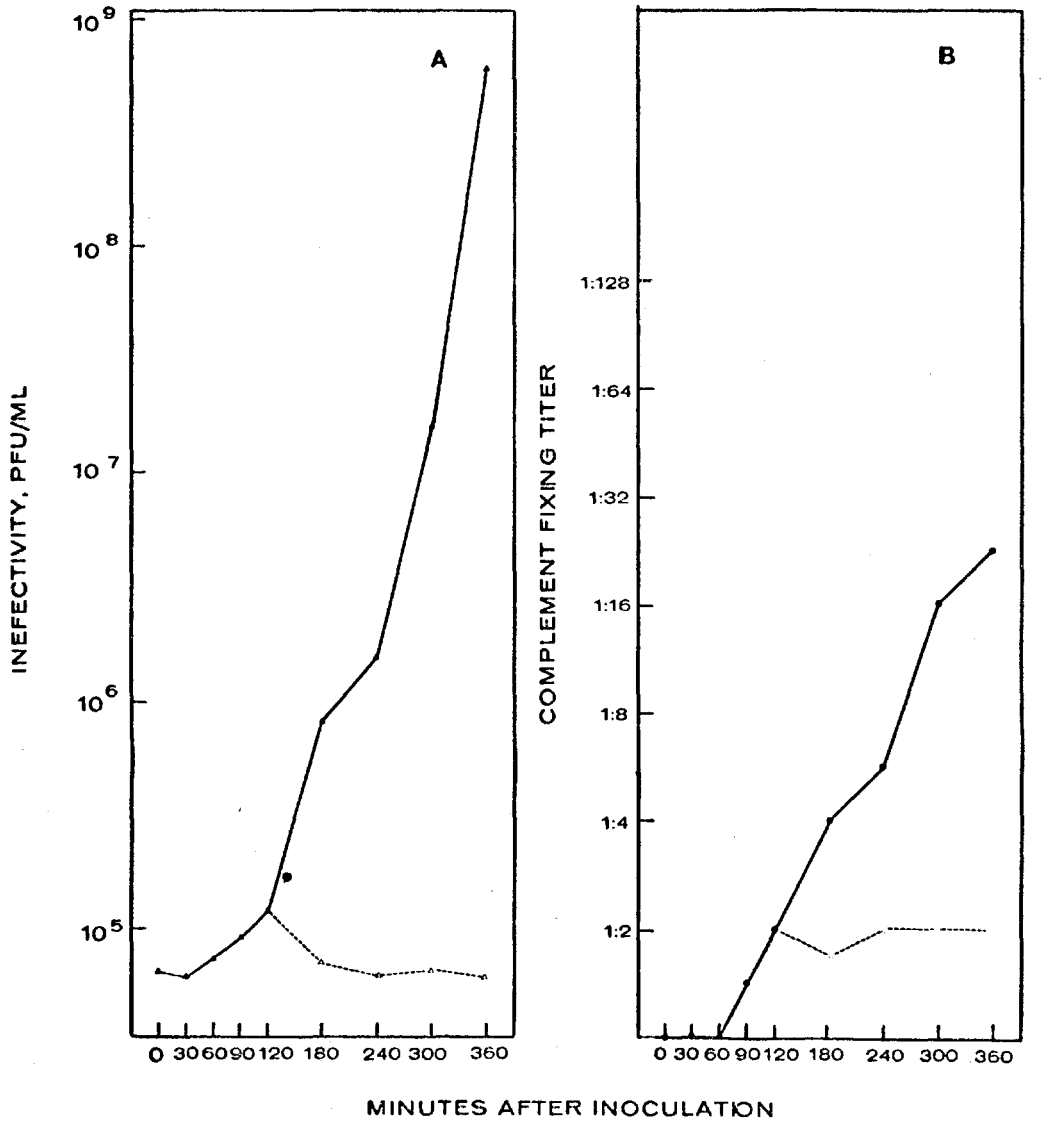


Fig. 13. Effect of cycloheximide on PFU and CF activity. L-cells were infected with VSV as in Fig. 12. At 2 hours post-infection the culture was split in two and cycloheximide (20 µgm/ml) added to one portion. Samples were removed from the cultures at the indicated times and after freezing and thawing, PFU activity (A) and CF activity (B) were determined. Solid line-control culture; broken line-cycloheximide added.

of the known VSV components.

For this study, viral components were radioactively labelled by growth of virus in medium containing ^{14}C-amino acids and the yield partially purified by differential centrifugation as described in Methods. The 41,000 x g pellet (virus pellet) was suspended in PBS, sonically treated for 15 seconds with a Biosonik III (lowest setting) microprobe to disaggregate the virus, and layered on 30 ml of a 5-30% sucrose gradient in PBS. After centrifugation for 1 hour at 4°C and 41,000 x g, successive 1 ml fractions of the gradient were collected by using an Isco model D fractionator. An aliquot of each fraction was used to determine PFU, CF, and radioactivity. The distribution of these activities along the gradient is shown in Fig. 14. Closest to the bottom of the gradient is the thin, visible band which corresponds to fractions 6 and 7 of the gradient. Associated with this band is some CF activity, a peak of radioactivity, and almost all the viral infectivity present in the gradient. This then is the band of infectious B particles as previously described. Higher up the gradient in fractions 11 and 12 is the LT particle band. This band shows CF activity and a peak of radioactivity. Some infectivity associated in this region may well be due to incomplete separation of infectious B particles. Material containing CF activity and radioactivity is also present at the top of the gradient depicted in Fig. 14.

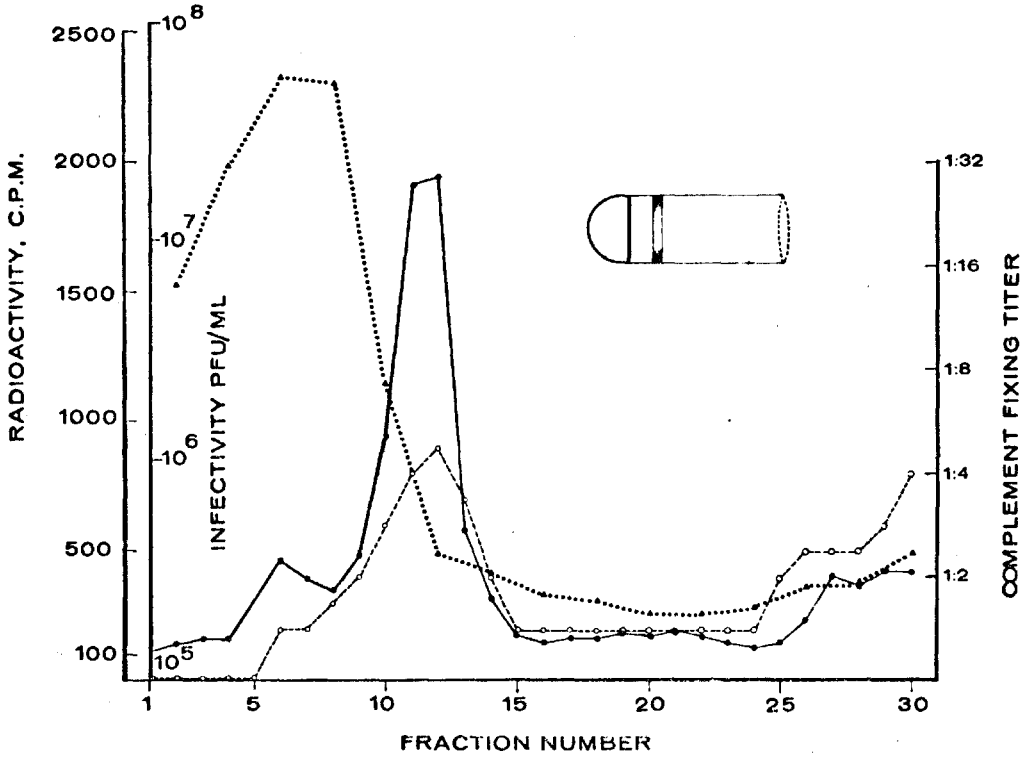


Fig. 14. Sucrose gradient purification of B and LT particles. VSV was grown in L-cells in the presence of ^{14}C-amino acids as described in text. After removal of cells by centrifugation, the cell-free fluid was centrifuged at 41,000 x g for 2 hours to pellet the virus. The viral pellet was resuspended in PBS, sonicated and layered on 30 ml of a 5-30% sucrose gradient in PBS. Centrifugation was at 41,000 x g for 1 hour. Successive 1 ml fractions were collected and samples removed for PFU, CF and radioactivity determinations. The orientation of the tube and the visible bands in the gradient are shown in the inset. The direction of sedimentation was from right to left.
Infectivity (▲), CF activity (o), Radioactivity (●

6. Protein Composition of "B", "LT" and "ST" Particles

In order to identify and characterize the structural proteins of the virus particles, purified B, LT and ST particles labelled with ^{14}C-amino acids were disrupted and analyzed on polyacrylamide gel as has been described in Methods.

The resultant distribution of radioactivity along the polyacrylamide gel for each particle is shown in Fig. 15. At least four major peaks of radioactivity and one or more minor peaks are seen in all "B", "LT" and "ST" particle preparations. Starting with the higher molecular weight protein, fractions are numbered VP_1, VP_2, VP_3 and VP_4 in order of increasing mobility. The minor peak or peaks between VP_3 and VP_4 are not consistent in all preparations, however, the possibility of these being structural proteins will be considered in the Discussion. Quantitative differences in the relative amount of radioactivity in any of the protein peaks between B and two different T particles were not reproducible.

Coelectrophoresis of a mixture of B and LT or ST particle proteins yield the same pattern shown in Fig. 15, thus proving that the electrophoretic mobility of the proteins from both B and T particles are identical.

The possibility remains that some of the viral proteins are not recovered in the gel. This was examined in two ways. First, electrophoresis of viral proteins under

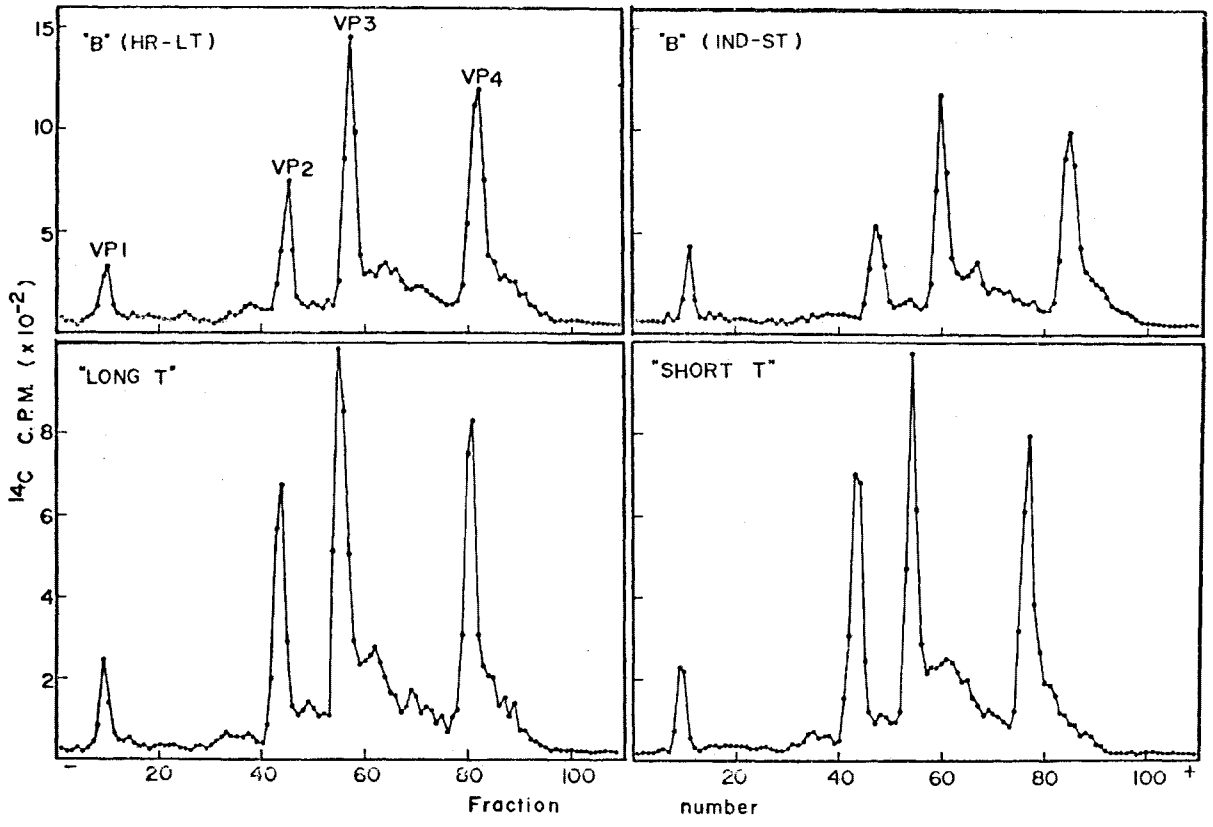


Fig. 15. Polyacrylamide gel analysis of B- and T- particle proteins.
B, LT and ST particles containing ^{14}C-labelled proteins were purified as shown in Fig. 10. The virus particles after pelleting at 41,000 x g for 2 hours were resuspended in alkylating buffer, followed by dialysis against 1000 volumes of dialysis buffer to degrade the particles to their constituent proteins as described in Methods. The samples were placed on 7.5% vertical polyacrylamide gels (17 cm in length and 0.9 cm in diameter) and run for 20 hours at 4 volts/cm gel. The gels were then sliced into 1.2 mm discs and radioactivity determined using Bray's scintillation fluid and a Beckman scintillation counter. The sample was applied at the cathodic side (to the left in these drawings). Upper two graphs show proteins of B particles from HR-LT strain and IND-ST strain; lower two graphs show proteins of "long T" and "short T" particles. Proteins are labelled VP_1, VP_2, VP_3 and VP_4 in order of increasing mobility.

conditions of reversed polarity showed that in this case no detectable radioactivity entered the gel. Secondly the recovery within the gel of the radioactivity applied was calculated. Under our conditions of gel slicing and subsequent counting of radioactivity, there was a loss of radioactivity owing either to quenching or to self-absorption in the gel. To determine the proper correction factor, a known amount of radioactively labelled protein sample was mixed uniformly with acrylamide. After the gel has solidified into a cylinder approximately 1 cm long, the gel was sliced and counted in the usual manner. From this experiment, it is determined that the counting efficiency for radioactivity in the gel slices was 12% of that obtained by the filter paper methods.

Using this correction factor, over 90% of the applied radioactive material is accounted for in the gel. On the basis of these results it is concluded that no major differences exist in the protein composition of B and T particles.

The molecular weights of four viral structural proteins were estimated in order to determine what proportion of the viral genetic information is used to specify the structural proteins of the virion.

7. Molecular Weights of Viral Proteins

Rather precise estimates of molecular weights can be obtained by comparing the mobility of proteins on polyacrylamide gels containing SDS with the mobility of standard proteins of known molecular weight. Under the proper experimental conditions the logarithm of the molecular weight is proportional to the mobility of the protein (Shapiro et al., 1967; Weber and Osborn, 1969).

Fig. 16A, B, C and D represent photographs of standard gels obtained after coelectrophoresis of viral proteins with various combinations of 18 different protein markers under different conditions of gel concentration and extent of cross-linking. A summary of the protein markers used and their molecular weights is presented in Table 1. Using the value for mobility determined from the gels pictured in Fig. 16, a curve of mobility versus the logarithm of the known molecular weights was plotted for each set and the result is shown in Fig. 17. As can be seen, only under one set of conditions - using a 7.5% gel with 0.2% cross-linking - was there proportionality over the entire range of molecular weights. Since such a wide variety of standards was used however, consistent estimates of the molecular weights of the viral proteins from their mobility could be obtained from all of the gel conditions. Accordingly the molecular weights of VP_1, VP_2, VP_3 and VP_4 are estimated at 190,000, 69,000, 50,000 and 29,000

Table 1

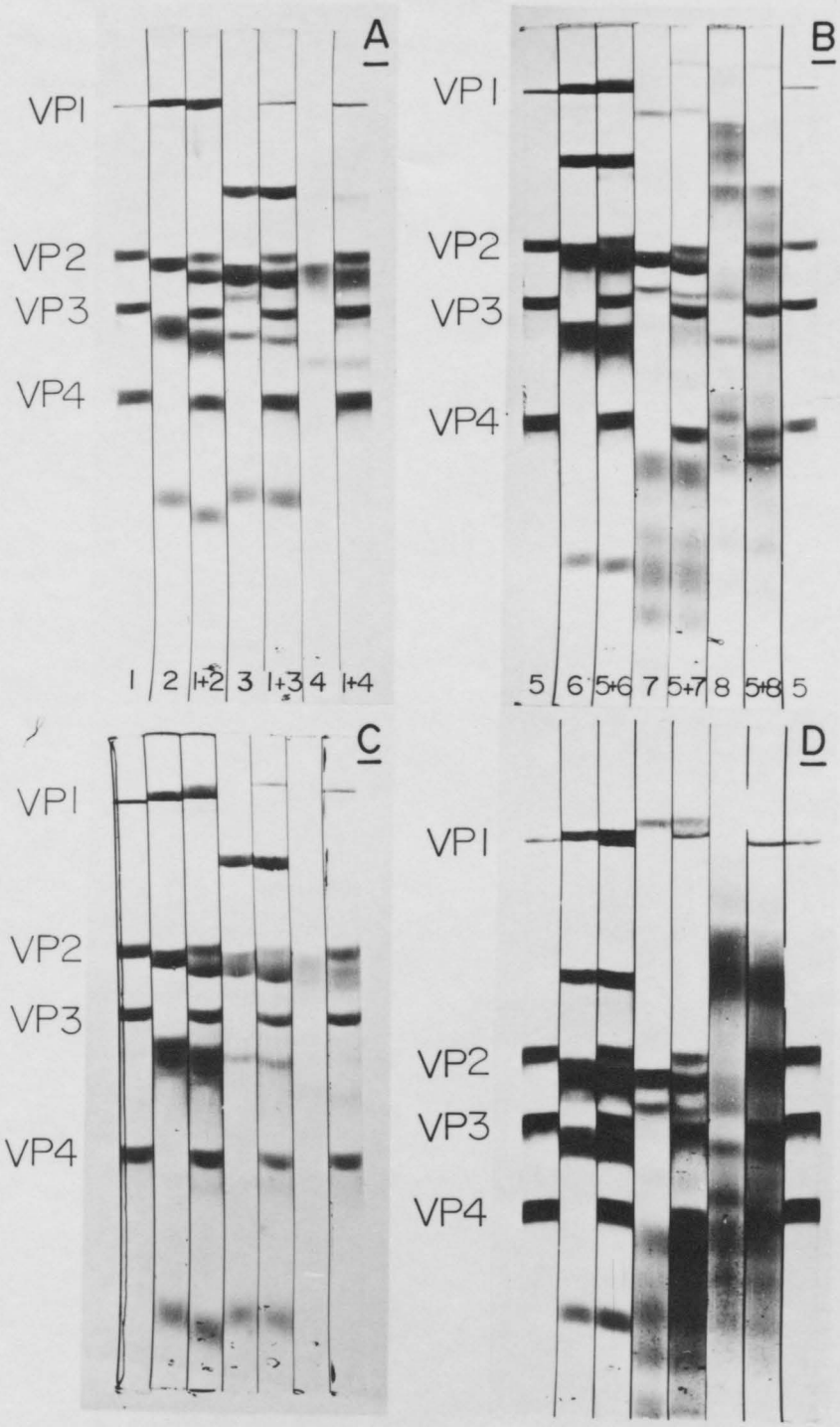
This Table Lists Molecular Weights of the Polypeptide Chains Taken from the Literature

Marker No†	Protein marker	Mol. Wt. of Polypeptide Chain	Reference
1	Myosin*	210,000	Slayter and Lowey, 1967; Gazith et al., 1970
2	Phosphorylase b*	100,000	Madsen, 1971; Cohen, 1971
3	γ-globulin G* (HL_2)	160,000	Edsall, 1953
4	Urease*	83,000	Svedberg and Pederson, 1940
5	γ-globulin HL chain	75,000	Rutishauser et al., 1968
6	BSA	66,000	Creeth, 1958; Edsall, 1953
7	Catalase*	60,000	Schroeder et al., 1964; Sund et al., 1967
8	Pyruvate kinase*	57,000	Steinmetz and Deal, 1966
9	γ-globulin H chain*	50,000	Rutishauser et al., 1968
10	Alkaline phosphotase*	50,000	Engström, 1961
11	Aldolase*	40,000	Kawahara and Tanford, 1966; Morse et al., 1967; Castellino and Barker, 1968
12	Pepsinogen	40,400	Williams and Rojagopalan, 1966
13	Pepsin	35,500	Edsall, 1953; Williams and Rojagopalan, 1964
14	Chymotrypsinogen	25,700	Weber and Osborn, 1969
15	γ-globulin L chain*	25,000	Edsall, 1953
16	Trypsin	23,300	Edsall, 1953; Weber and Osborn, 1969
17	Hemoglobin	15,500	Weber and Osborn, 1969
18	Cytochrome c	13,400	Edsall, 1953; Atlas and Faber, 1956

† The same numbers are used to identify the protein in Fig. 17.

* Proteins which under native conditions exist as oligomers.

Fig. 16. Polyacrylamide gel electrophoresis of VSV proteins and 18 different marker proteins.
The proteins were electrophoresed in 7.5% gel with high (A), normal (B) and low (C) cross-linker as well as 5% gel with normal cross-linker (D). The samples in group A are the same as in group C; those in group B are the same as in group D.
The proteins of each gel are: (1) virus; (2) myosin, catalase, aldolase, and hemoglobin; (3) phosphorylase b, pyruvate kinase, pepsinogen and cytochrome c; (4) γ-globulin G, BSA, and pepsin; (5) virus; (6) myosin, phosphorylase b, BSA, pyruvate kinase, aldolase and hemoglobin; (7) catalase, alkaline phosphotase, pepsin, trypsin and cytochrome c; (8) γ-globulin G, urease, pepsinogen, and chymotrypsinogen.
Each set of gels were run independently of each other. Gels were stained with Coomassie Blue.


A
VP1
VP2
VP3
VP4
1 2 1+2 3 1+3 4 1+4
B
VP1
VP2
VP3
VP4
5 6 5+6 7 5+7 8 5+8 5
C
VP1
VP2
VP3
VP4
D
VP1
VP2
VP3
VP4

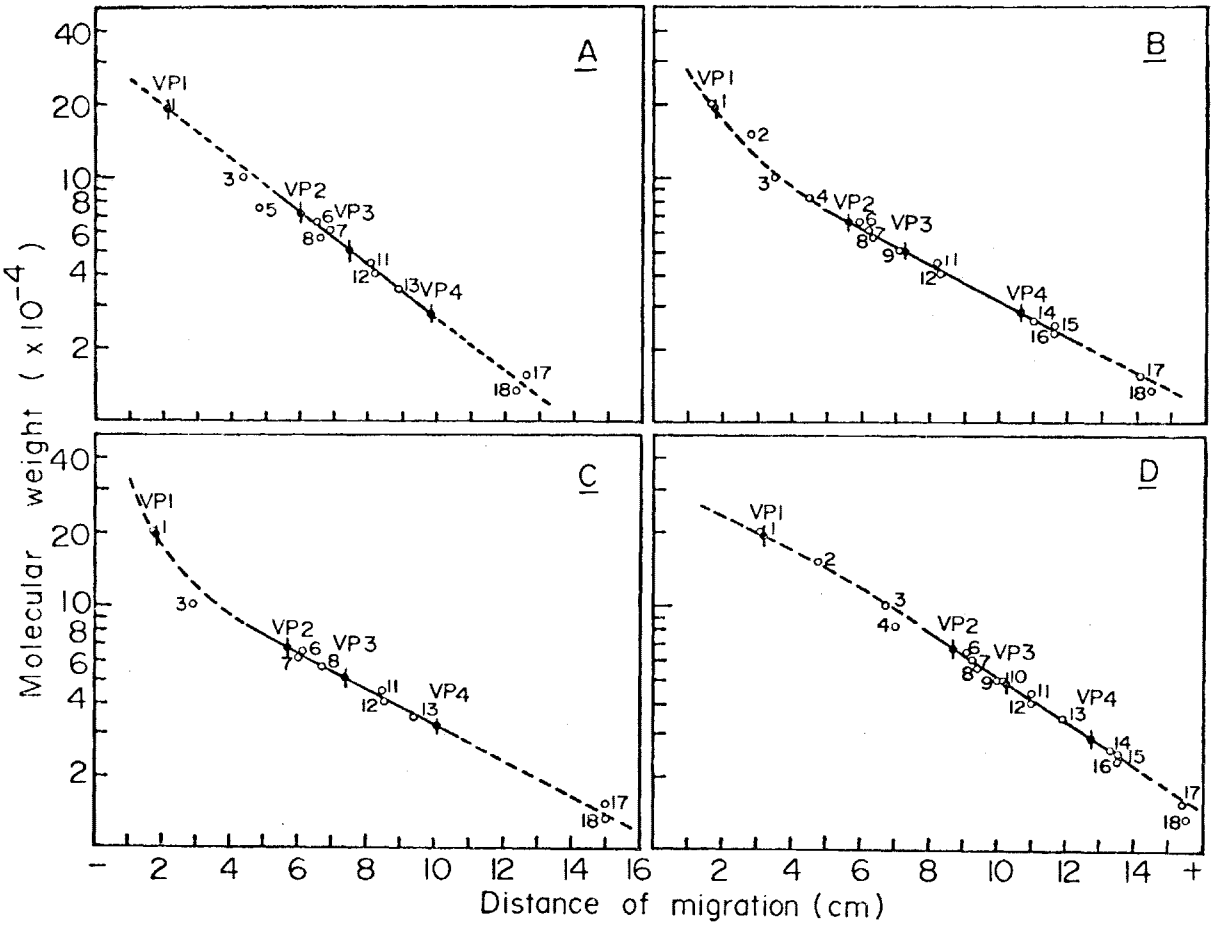


Fig. 17. Determination of molecular weights of the VSV structural proteins using 18 standard marker proteins. The molecular weight of each marker is taken from Table 1 and electrophoretic mobilities are from Fig. 16. The number of reference proteins are the same as those in Table 1.

respectively. The excellent agreement between these results and those determined from sedimentation velocity values (presented in Chapter 23) should be noted.

Electrophoresis in polyacrylamide gel afforded a relatively simple and reliable procedure for the identification of a particular virus protein. Since VSV is composed of a nucleoprotein and an outer envelope we were now in a position to determine which particular proteins were associated with each of these structures.

8. Purification of Viral Nucleoprotein Cores and Virus Coat

Nakai and Howatson (1968) described the release of viral cores from B and T particles treated with sodium deoxycholate (DOC). DOC treatment of virus followed by sucrose gradient centrifugation enables one to obtain pure particles from either B or LT particles. Fig. 18 b and c shows that DOC treatment of B particles or LT particles produce components separable from each other by rate zonal centrifugation and are characteristic of the virus particles from which they were produced. These are nucleoprotein cores and contain RNA having the same sedimentation coefficient as RNA extracted from the parent virus particle (Petric and Prevec, 1970). Linear interpolation of sedimentation coefficients on sucrose gradients using published sedimentation coefficients of cellular polyribosomes as standards (Noll, 1967; Petric and Prevec, 1970) gives values of 100S and 140S

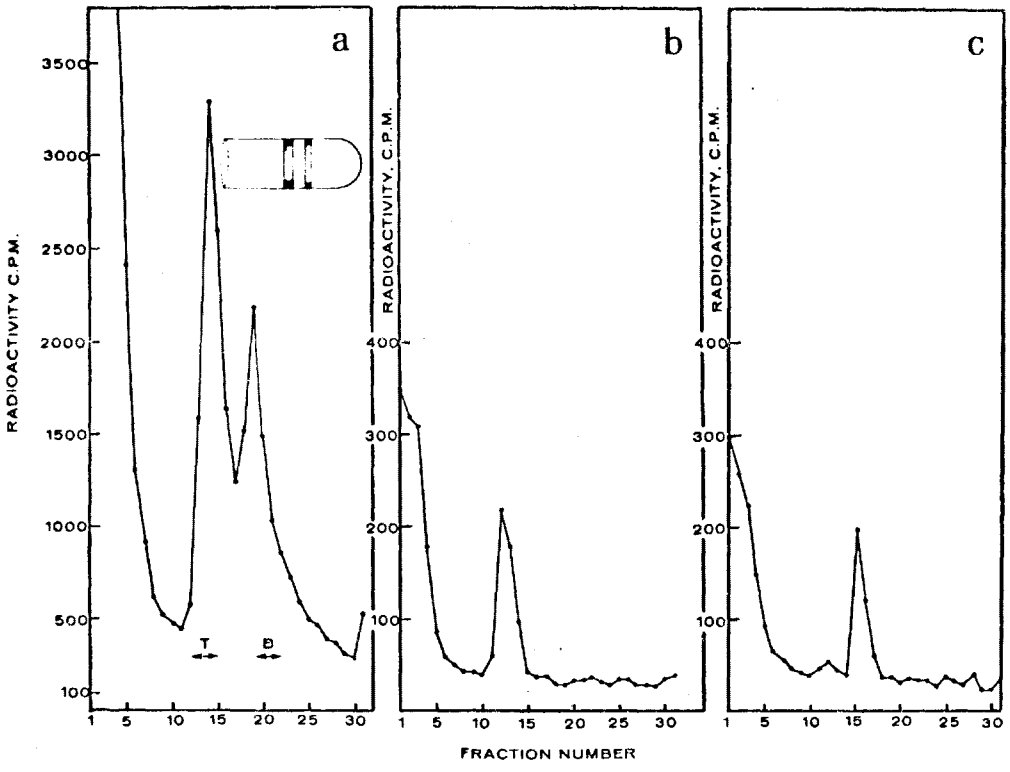


Fig. 18. Purification of nucleoprotein cores from B and LT particles.

B and LT particles were prepared as described in Fig. 10. Centrifugation on a 5-30% sucrose gradient in PBS at 41,000 x g for 1 hour resulted in the radioactive peaks shown in a. B particles and T particles were taken from fractions indicated by the bars in a and after pelleting by centrifugation at 41,000 x g for 2.0 hours were each separately resuspended in 0.5% DOC and 0.5% BRIJ in PBS. After treatment for 1 hour at room temperature the material was layered on 5-30% sucrose gradients in PBS and centrifuged at 51,500 x g for 2.5 hours. This resulted in the purification of LT particle cores with sedimentation coefficient of 100S (b) and B particle cores of 140S (c) from the dissociated strip or coat proteins of each seen at the top of the gradient tube. The top of the gradient tube is to the left in all diagrams.

for LT particle cores and B particle cores respectively. It should be pointed out that DOC treatment of virus is relatively inefficient in the production in these cores.

The radioactivity at the top of the gradients shown in Fig. 18 b and c presumably represents viral envelope protein and perhaps some core protein arising from complete disruption of a small percentage of virus particles. This protein fraction, obtained after DOC treatment of virus shall be referred to as the viral coat protein.

9. Proteins of Viral Core and Coat

The purified radioactive core and coat protein fractions were analyzed by polyacrylamide gel electrophoresis in a manner analogous to that described for whole viral particles. As a control marker for protein mobility in all subsequent experiments, virus protein obtained from virus partially purified by differential centrifugation only was used. This marker shows a protein pattern on polyacrylamide gel qualitatively the same as that shown in Fig. 15 and shall be referred to as "total virus protein".

It was first necessary to ensure that treatment with or the presence of DOC in virus protein extracts did not in itself affect the mobility of the virus protein on polyacrylamide gels. In this experiment, a small portion of the total virus sample was incubated with DOC under conditions identical to those used to prepare core and coat protein.

Without further fractionation, the material was then treated with SDS, urea, acetic acid and mercaptoethanol, and analyzed on polyacrylamide gels. A parallel non-DOC-treated sample was also analyzed. No difference in protein mobility as a result of the DOC treatment is seen by comparing Fig. 19 a with 19 a'. This experiment ensured that preparation of core protein and coat protein by DOC treatment would have no effect on the electrophoretic mobility of these proteins.

Core and coat proteins were prepared by DOC treatment of purified B and T particles followed by purification on sucrose gradients. Fig. 19 b shows the distribution of ^{14}C-radioactivity on polyacrylamide gel obtained from the electrophoresis of 60,000 counts/minute of disrupted core protein. Only one electrophoretically homogeneous species of protein was observed. To determine which viral protein corresponded to core protein, 60,000 counts/minute of disrupted core protein and 40,000 counts/minute of total protein were coelectrophoresed. The total protein was identical to that used to obtain Fig. 19 a (note changes in radioactivity scale). The coelectrophoresis resulted in a large increase in radioactivity associated with virus protein peak number 3 (Fig. 19 b'). It is therefore concluded that VP_3 arises from the protein of the viral nucleoprotein core.

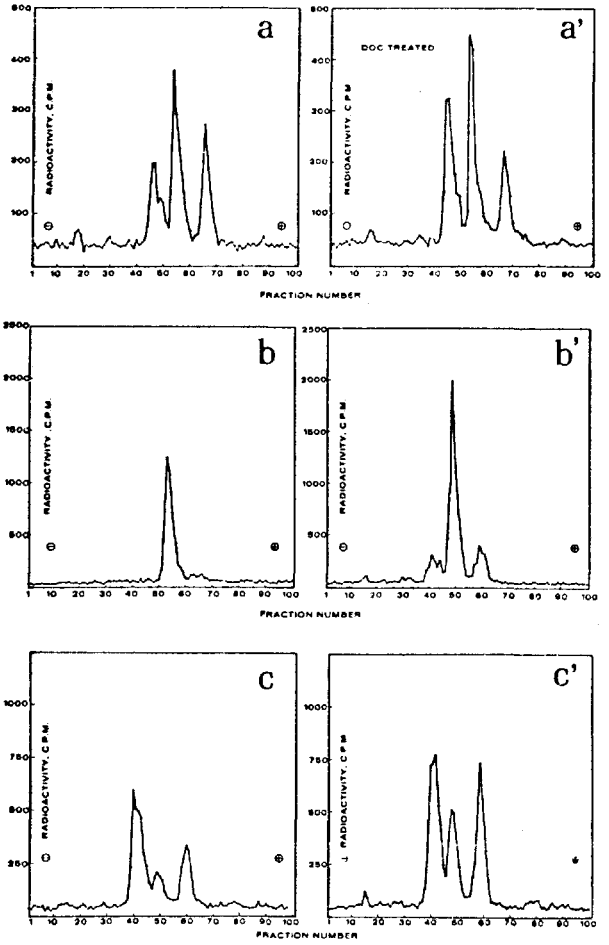


Fig. 19. Polyacrylamide gel analysis of VSV core and coat protein.
Radioactive total virus (obtained by using the pellet of the 41,000 x g centrifugation described in Fig. 14) was used as a mobility standard in these experiments. Fig. a shows the radioactive profile on acrylamide of 40,000 cpm of disrupted total virus protein. In a' is shown an identical sample exposed to 0.5% DOC and 0.5% BRIJ prior to disruption. Viral core and strip proteins were prepared and purified as shown in Fig. 18. Electrophoresis of 60,000 cpm of disrupted core protein gave the pattern shown in b while a mixture of 60,000 cpm of core protein and 40,000 cpm of total protein gave the pattern shown in b'. Similarly the pattern obtained from 60,000 cpm of disrupted strip protein (c) and a mixture of 60,000 cpm of strip protein plus 40,000 cpm of total protein (c') is shown. Electrophoresis was done in 5% polyacrylamide gel for 19 hours from left to right.

In a similar manner, the coat protein fraction obtained after DOC treatment of whole virus was examined. Two major peaks of radioactivity with a less highly radioactive peak between them was obtained (Fig. 19 c). By coelectrophoresis with total protein (Fig. 19 c') it can be seen that the major peaks corresponded to VP_2 and VP_4. Some protein moving with the electrophoretic mobility of VP_3 was also present in the coat protein fraction. Whether this represents a species different from that found in viral core or whether it results from contamination of coat protein by core material disrupted by DOC treatment is not clear.

It was noted, however, that the amount of protein VP_3 relative to proteins VP_2 and VP_4 present in coat protein preparation is variable, suggesting that the second explanation may be more likely. From these experiments it is concluded that VP_3 comes from the core of the virus, whereas VP_2 and VP_4 come from the viral coat or envelope. VP_1 was not detected in either core or coat protein preparations so its physical location within the virus remains unknown.

While DOC treatment of virions results in the dissociation of ribonucleoprotein cores and the two proteins of the virus coat, Cartwright et al. (1970) showed that the treatment of virus with the non-ionic detergent Nonidet P_{40} dissociated only the protein VP_2 from the virion complex.

This result was substantiated by the following experiment. B particles were prepared and purified by sucrose gradient centrifugation. The virions were then treated with Nonidet P_{40} at a final concentration of 0.2% and the resultant material again centrifuged on a 15-30% sucrose gradient in PBS at 81,000 x g at 5°C for 40 minutes. Some UV absorbing material remained at the top of the gradient while the remainder moved to the bottom of the gradient. Material from both the rapidly and slowly sedimenting fractions was dissociated by the usual procedure and examined by polyacrylamide gel electrophoresis. Since a sufficient amount of virus protein was present the gels were stained with Coomassive blue for viral examination. As seen in Fig. 19-1, the slowly sedimenting fraction contains only VP_2 while the rapidly sedimenting "skeleton" fraction contains VP_1, VP_3 and a small amount of VP_4. The apparent loss of VP_4 after treatment with Nonidet P_{40} was also observed by Cartwright et al. (1970) and remains to be explained.

As described in the Introduction, the infection of cells in culture with VSV results in the release, into the extracellular fluid, of two smaller antigens characterized by their sedimentation coefficient of 20S and 6S in addition to B and T particles (Bradish et al., 1956; Brown and Cartwright, 1966; Brown et al., 1966). These smaller antigens have been studied immunologically by Grown and his co-workers but little

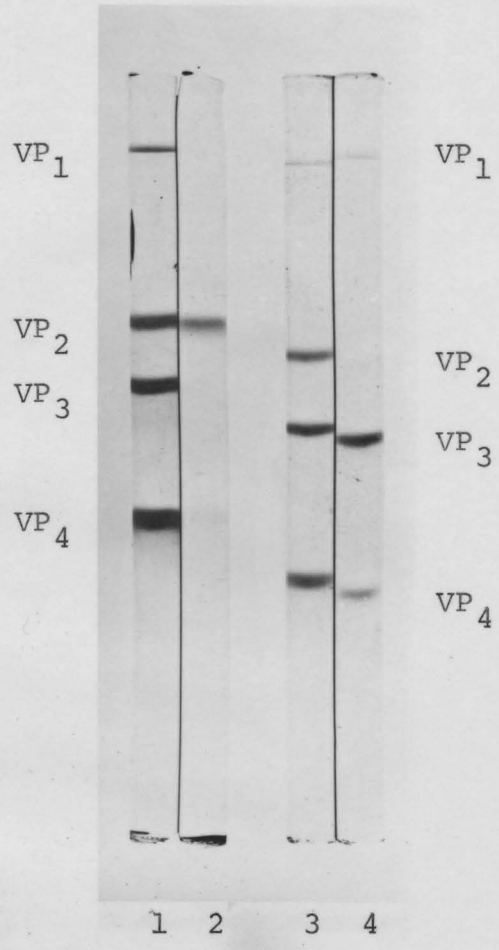


Fig. 19-1. Proteins from Nonidet P_{40} treated virions. 1) VSV; 2) Slowly sedimenting fraction from Nonidet P_{40} treated virus; 3) VSV; 4) Skeleton separated from Nonidet P_{40} treated virus. The proteins were separated in 7.5% polyacrylamide gel and gels were stained with Coomassive blue.

is known of their functional origin or significance.

Since these soluble antigens contribute approximately 65% of the total CF activity in lysate of infected culture, it was important to attempt to understand their origin and function. Accordingly, the radioactively labelled viral proteins in the virus free supernatant fraction of an infected cell lysate which contain 20S and 6S antigen was precipitated with 60% saturated ammonium sulphate and treated for acrylamide gel analysis as described in Methods.

10. Proteins of 20S and 6S Antigens

Fig. 20 shows the polyacrylamide gel pattern of combined 20S and 6S antigens. It is evident that there are two distinct proteins in the supernatant of the cell-free, virus-free lysate. In order to determine the origin of each, the radioactive viral proteins in the supernatant were further purified. A volume of 1 ml of the ammonium sulphate precipitated protein concentrate was layered on a 5-30% sucrose gradient in PBS and centrifuged for 16.5 hours at 51,000 x g and 4°C on a Spinco SW50 rotor. Successive 0.33 ml fractions were collected by dripping the gradient through a hole made in the bottom of the centrifuge tube and each fraction was subsequently analyzed for CF activity and ^{14}C radioactivity (Fig. 21). A peak of CF activity and radioactivity is present approximately half way down the gradient. This corresponds to the 20S antigen (Bradish et al., 1956). Material having

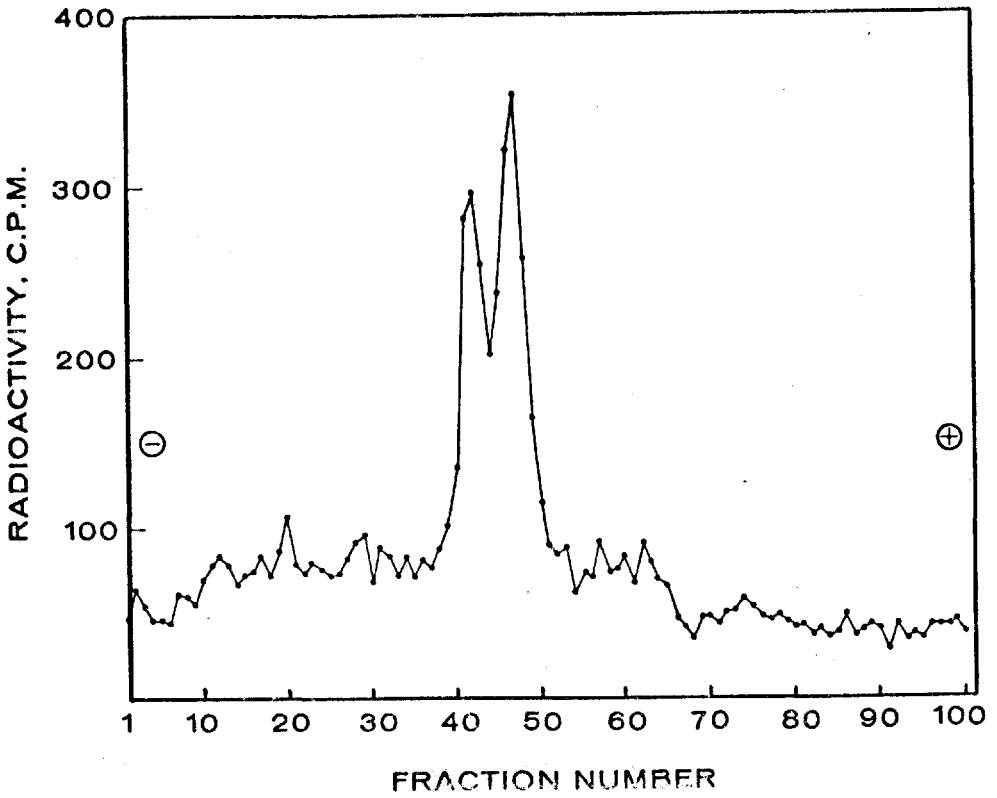


Fig. 20. Polyacrylamide gel analysis of combined 20S and 6S antigens.
Radioactive free protein containing both 20S and 6S antigens prepared by ammonium sulphate precipitation of the 41,000 x g supernatant as described in Methods. The precipitate was dialyzed, concentrated by flash evaporation and the protein disrupted with SDS, urea, acetic acid and mercaptoethanol as described in Methods. Application of 35,000 cpm of protein followed by electrophoresis for 15 hours at 3 volts/cm yielded the above radioactive distribution in the 5% polyacrylamide gel. Electrophoresis was from left to right.

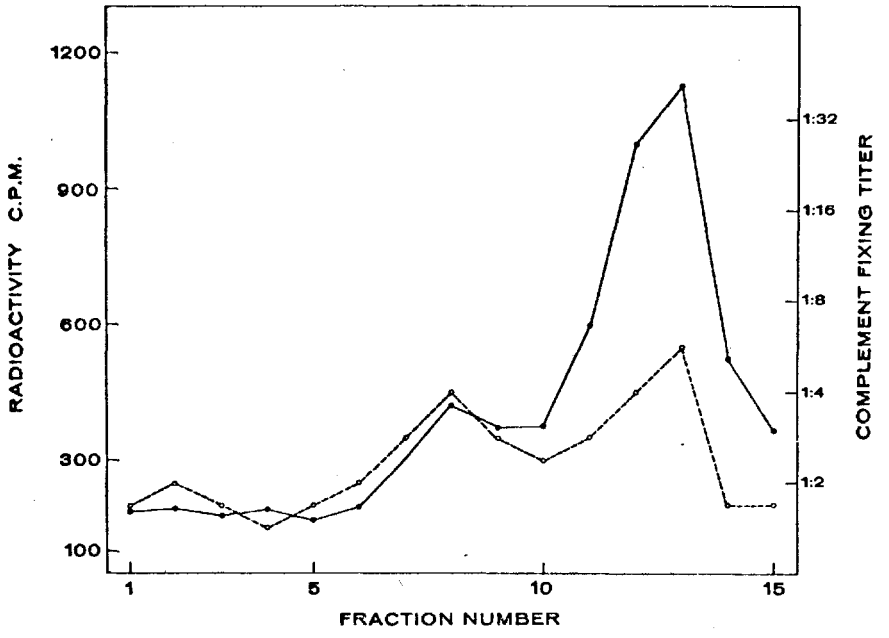


Fig. 21. Sucrose gradient purification of 20S and 6S antigen.
Radioactive virus was grown and partially purified as described in Fig. 14. The 41,000 supernatant was made 60% saturated in ammonium sulphate and the resultant precipitate centrifuged and washed as described in the text. The washed pellet was resuspended in 1 ml of PBS, dialyzed against PBS and 0.2 ml layered on a 5 ml 5-30% sucrose gradient in PBS. Centrifugation was at 51,000 x g for 16.5 hours. Successive 20 drop fractions were collected through the bottom of the tube and a portion of each used for determination of radioactivity (●) and CF activity (O). The bottom of the gradient tube is to the left.

both CF activity and radioactivity is also present at the top of the gradient tube and is originated the 6S antigen. It is possible that much of the radioactivity present at the top of the gradient, although it occupies the same region as 6S viral antigen, is in fact, due to contaminating cellular protein. As a control for this possibility ^{14}C labelled fractions from uninfected cells were prepared in an identical way to infected cultures. No label was present in the 41,000 x g pellet and although some radioactivity was present in the 41,000 x g supernatant fraction, none was precipitated with 60% saturated ammonium sulphate. The value of this particular type of control is dubious, since cells infected with virus are undoubtedly more fragile and capable of leaking their own proteins to the culture medium than are growing, uninfected cells.

From the above experiment, however, one may tentatively conclude that the radioactivity associated with 20S and 6S antigen did not result from nonviral material cosedimenting with these viral antigens.

Fig. 22 a shows that the purified 20S antigen contains one major protein component. The presence of 2 distinct peaks of lower mobility in this particular experiment was undoubtedly due to incomplete denaturation of structures, since in three other runs only the major protein peak was present. Coelectrophoresis of this material with total virus showed (Fig. 22 a') that the protein of the 20S antigen

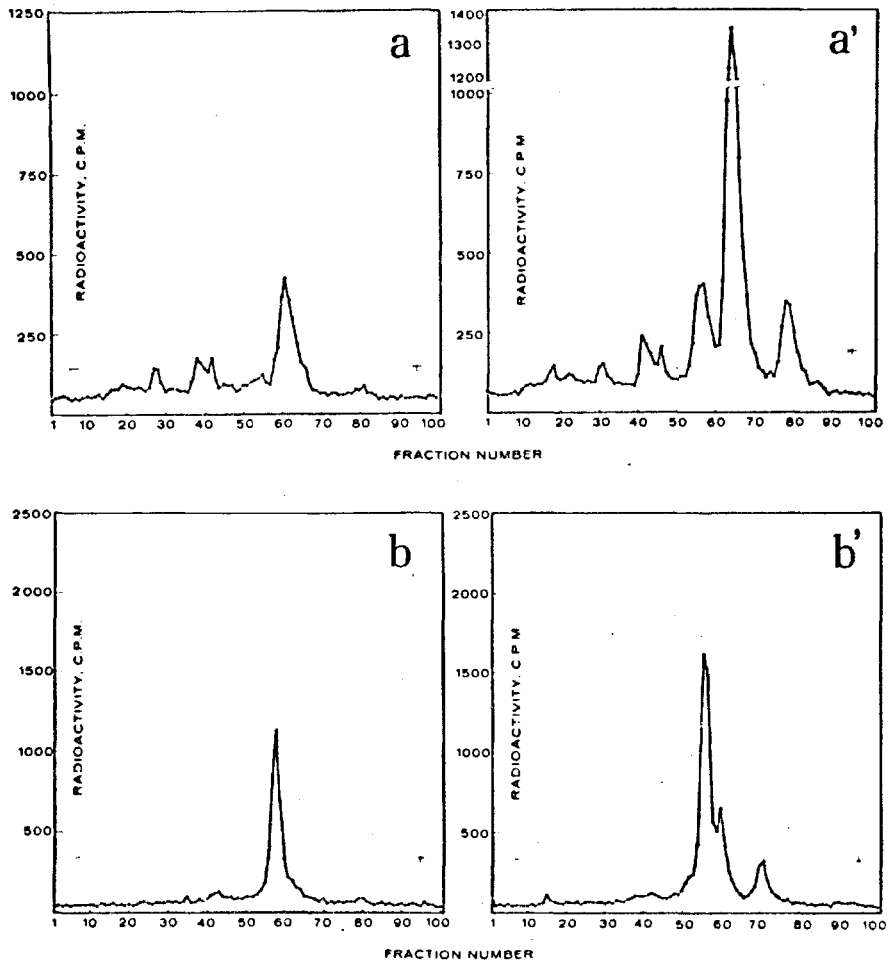


Fig. 22. Polyacrylamide gel analysis of 20S and 6S antigens. Radioactive 20S and 6S antigens were prepared and purified as shown in Fig. 21. Each isolated fraction after dialysis, concentration and disruption was analyzed by electrophoresis for 19 hours in 5% gel at 3 volts/cm. Total virus standards were identical to those used in Fig. 15. 54,000 cpm of disrupted 20S antigen (a), 54, 000 cpm of disrupted 20S antigen plus 60,000 cpm of total virus (a'), 60,000 cpm of disrupted 6S antigen (b), 60,000 cpm of disrupted 6S antigen plus 40,000 cpm of total virus.

corresponded to VP_3. The mobility of the protein from the 20S antigen is therefore identical to that of the protein from the viral core. Electrophoresis of the 6S viral antigen also yields a single protein (Fig. 22 b). On coelectrophoresis with total protein (Fig. 22 b'), the 6S protein has a mobility similar to that of virus protein VP_2.

Close examination of this result and the clear demonstration seen in the next chapter show that the protein of the 6S antigen is not identical to the virion protein VP_2 but seems to be displaced to the high mobility side of this virion protein.

From these experiments it is concluded that the 20S antigen possess a single protein with a mobility characteristic of the viral nucleoprotein fraction whereas 6S antigen also has a single protein with mobility similar to but probably not identical with viral protein VP_2.

The observation by Burge (personal communication and Burge and Huang, 1970) that virion protein VP_2 is a glycoprotein suggested yet another method to check the relationship of this protein and that of the 6S antigen.

11. Determination that the 6S Antigen is a Glycoprotein

Radioactive viral antigens were prepared by growing virus in the presence of ^{14}C-glucosamine and then purifying virions, 20S antigens, and 6S antigens on sucrose gradients. The radioactive glucosamine was found in virus particles and

in the 6S antigen; no label was detected in the 20S antigen. When the labelled virions and 6S antigen were treated with urea, SDS, and mercaptoethanol and examined on polyacrylamide gels with the use of ^{3}H-amino acid-labelled protein from virus particles as marker, the glucosamine in virus particles and in the 6S antigen was found to be associated with a component which migrates with a mobility slightly greater than that of VP_2 (Fig. 23).

In an effort to compare directly, the viral and 6S glucosamine-containing components, infected cells were labelled simultaneously with ^{14}C-glucosamine and ^{3}H-amino acids between 1 and 10 hours post-infection. Both virus particles and 6S antigen were purified by sucrose gradient centrifugation from the extracellular fluid. The 6S antigen was further purified by immune precipitation with virus anti-coat antibody. (The rationale for this particular step will be apparent only after the immunological relationships are described in the next section.)

Both virus and 6S antigen were disrupted and analyzed on polyacrylamide gels. In this analysis, the glucosamine label in the virus coincided exactly with the tritium protein label (Fig. 24 b). Both ^{14}C and ^{3}H radioactivity in the 6S antigen fraction were also coincident (Fig. 24 a), suggesting that protein and carbohydrates are associated in one glycopeptide. A comparison of the ratios of ^{14}C to ^{3}H in Fig. 24 a and 24 b indicates that the 6S antigen contains approximately twice as

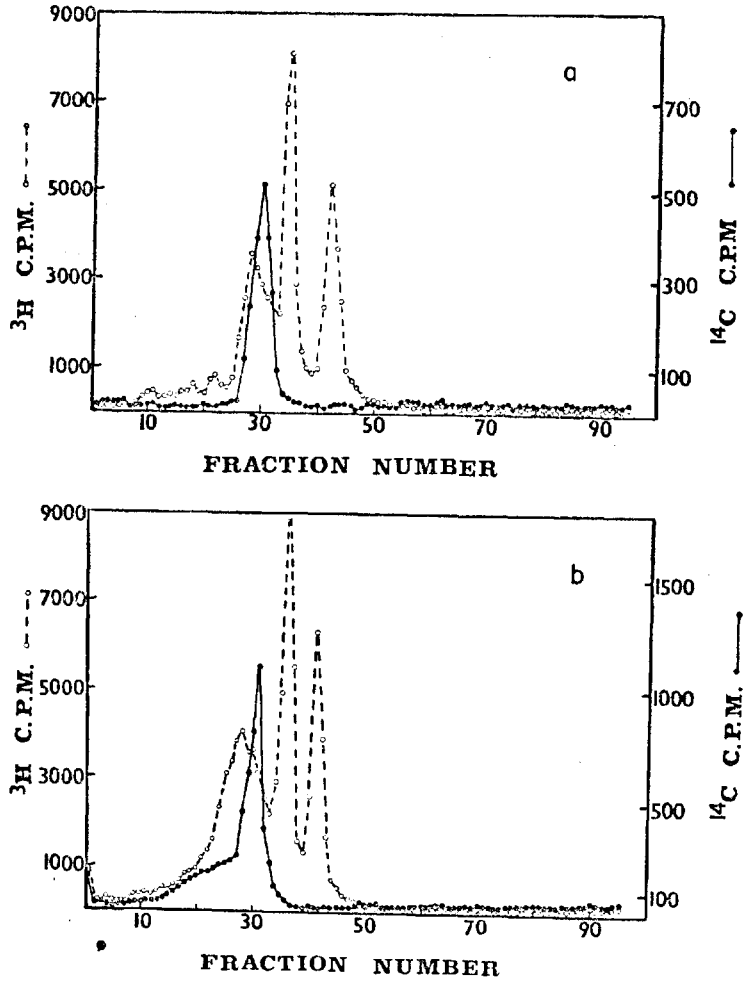


Fig. 23. Polyacrylamide gel analysis of glucosamine containing fraction from 6S antigen and mature virions. ^{14}C-glucosamine labelled virus lysate was prepared as described in Methods. Virus particles and free antigens were concentrated and purified on sucrose gradients. Both purified virus particles (a) and 6S antigen (b) were mixed with ^{3}H-labelled viral structural proteins as markers, denatured and examined by electrophoresis in 5% polyacrylamide gel.

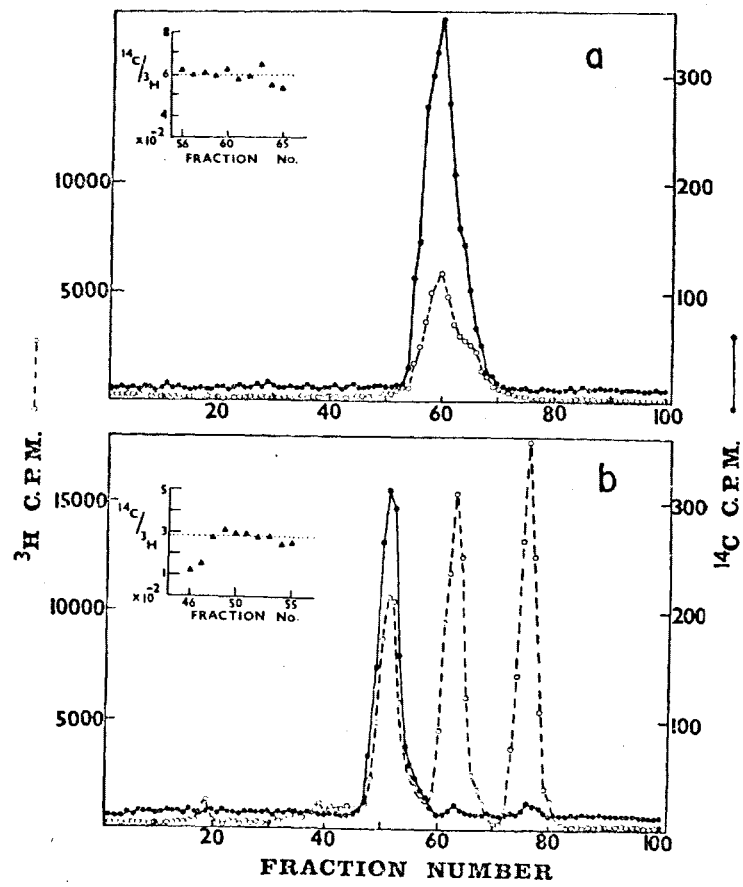


Fig. 24. Polyacrylamide gel analysis of ^{14}C-glucosamine and ^{3}H-amino acid labelled proteins in 6S antigen and mature virions.

Virus particles and free antigens labelled with ^{14}C-glucosamine and ^{3}H-amino acids were prepared and purified from a single lysate. Purified virus was taken directly from sucrose gradients whereas sucrose gradient purified 6S antigen was further purified by immune precipitation as described for Fig. 30. The dual labelled virus (b) and the 6S immune precipitate (a) were separately denatured and examined by electrophoresis in 5% polyacrylamide gel. The ^{3}H-radioactivity (O) and ^{14}C-radioactivity (●) were determined. In the inset in each figure is plotted the ratio of ^{14}C counts to ^{3}H counts for that region of the gel which contains ^{14}C-glucosamine radioactivity.

much glucosamine per unit of protein as is present in VP_2 of the virus.

12. Proteins of Prelabelled Cells

In addition to the virus-specific antigens, Cartwright and Pearce (1968) reported that VSV carries with it a cellular antigen, presumably arising from the cell membrane, which appears to form part of the viral envelope on emergence of the infectious particle from the cell. In order to determine if this cell-specific antigen may be a protein, the following experiment was conducted. L-cells were grown in the presence of ^{14}C-amino acids for a 24 hour period. The cells were then grown for a further seven hours in MEM in the absence of radioactive amino acids in order to clear any amino acid pools of radioactive precursors. The cells were then infected and virus harvested after 12 hours in the usual manner. Any radioactivity present in the viral components should be due primarily to cellular proteins incorporated into the particles. Total virus was prepared by centrifugation at 41,000 x g and the pelleted virus was purified by sucrose gradient centrifugation. Although intense light scattering bands (i.e., large quantities) of B and T particles could be visibly observed on the gradients, no radioactivity above background could be detected in either fraction. Since it is difficult to estimate the amount of radioactivity which might be contributed by a hypothetical cellular protein, it

cannot be absolutely stated that no cellular protein components are present in the virions. This possibility, however, seems remote.

While no radioactivity was observed in the virions, there is however, radioactive protein in the supernatant which can be precipitated with 60% saturated ammonium sulphate. This material was analyzed on polyacrylamide gels and, as seen in Fig. 25, consists of mainly heterogeneous material with one predominant peak of radioactivity. By comparison with other gels of total virus run under identical conditions at the same time, this peak appears to be located between VP_3 and VP_4. This cellular protein appears to be distinct from the viral proteins as previously identified, and it is concluded that, under these conditions, a cell specific viral carried protein is not present. Since there is good evidence that the carbohydrate portion of the glycoprotein in the virion may be specified by the infected host, (Grimes and Burge, 1971), it is possible that these residues constitute the host specific antigens in the virion.

The studies described thus far have suggested a number of similarities in protein composition of the different antigens produced by VSV infection. Since isolated viral antigens were available, in particular the viral nucleoprotein core and viral coat, such suggested relationships could be checked by procedures which determine the degree of immunological cross-reactivity between virus antigens.

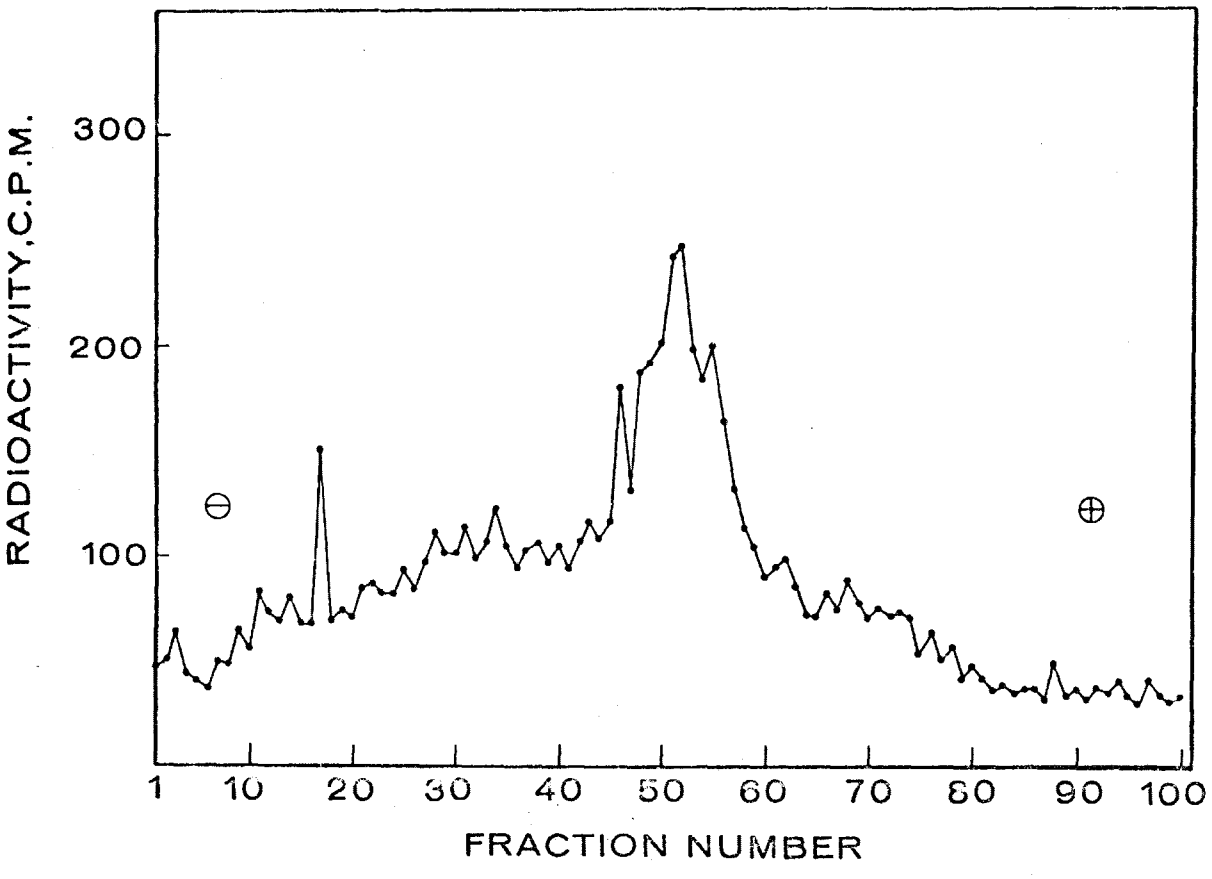


Fig. 25. Protein released from prelabelled infected cells. Cells were incubated with ^{14}C-amino acids for one generation time and for a further 7 hours in the presence of non-radioactive growth medium. They were then infected with virus at a moi = 50 and the viral fluid was harvested. The only radioactive protein present in the cell-free fluid was precipitated from the 51,000 x g supernatant with ammonium sulphate and prepared for electrophoresis. Electrophoresis was done in 5% gel for 15 hours at 3 volts/cm from left to right.

13. Cross-Reactivity of 20S and 6S Antigens with Virion Proteins

Antibodies to the isolated ribonucleoprotein core and separately to the virion coat components were prepared in rabbits. The cross-reactivity, as determined by CF of each of these antisera to the 20S and 6S antigens was checked as follows: The lower molecular weight antigens present in cell-free, virus-free lysates of the Indiana serotype were concentrated by ammonium sulphate precipitation fractionated on sucrose gradients. Successive 1 ml fractions were collected, and a portion of each was separately tested for CF activity with the appropriate antiserum. The optical density profile along the gradient shown in Fig. 26 is due to components of fetal calf serum present in lysate fluids; the absorbance peaks probably represent the 7S and 19S immunoglobulin fractions.

When antibody to virus coat was used to check the fractions for CF activity, the only significant activity was found in the 6S region of the gradient. This result provides evidence that the 6S antigen is immunologically similar to one of the antigens in the virus coat. It also shows that the 20S viral antigen is antigenically unrelated to the components of the virus coat. On the other hand when antibody to virus ribonucleoprotein was used to check the fractions there was considerable CF activity in a region of the gradient having a sedimentation coefficient about 20S (fractions 17 and 18). The heterogeneity of CF activity suggests that a number of

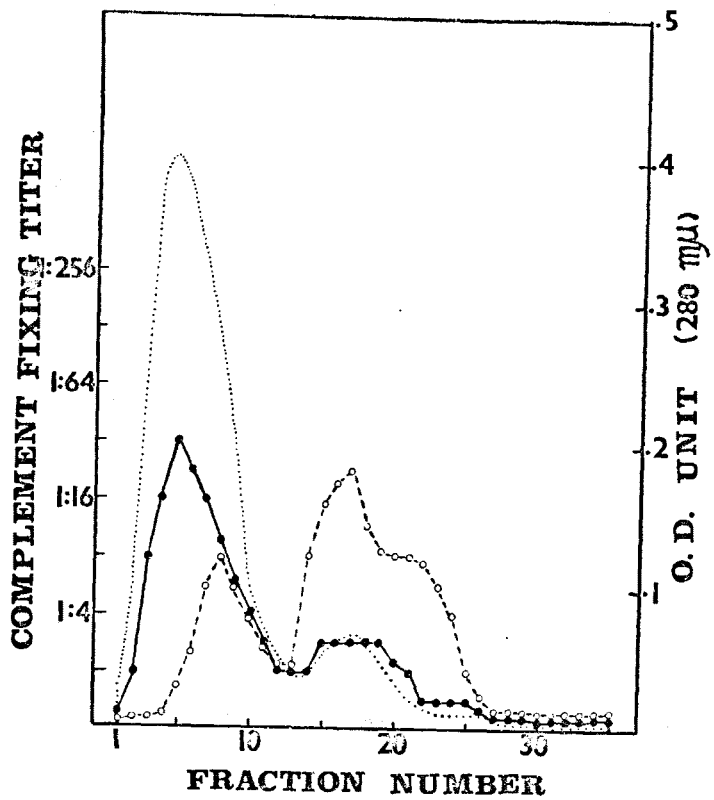


Fig. 26. Complement fixing activity of Indiana 20S and 6S antigens with homologous coat and core antibody.
The low molecular weight antigens were prepared from virus lysates by differential centrifugation and ammonium sulphate precipitation and fractionated on sucrose gradients. The optical density (280 mμ) was monitored continuously (....) and 1.0 ml gradient fractions collected. The top of the gradient is to the left. The CF activity of each gradient fraction was tested against Indiana core antibody (O) and Indiana coat antibody (●). Four units of antibody, with four units of complement and one unit of hemolysin were used in a microtiter CF test as described by Sever (1962).

distinct structures may be present in this region of the gradient. Somewhat unexpectedly, antibody to virus core also showed some CF activity with material in the leading edge of the 6S antigen region (fraction 7 to 10). Subsequent experiments to repeat this particular effect were unsuccessful and it is believed that the result in this particular experiment is an artifact created by freezing and thawing of the viral lysate. In any case, the result presented shows that the 20S antigen is related antigenically to the material of the ribonucleoprotein core.

14. Immunological Comparisons of Antigens of both Indiana and New Jersey Serotype

As stated in the Introduction, Myers and Hanson (1962) have shown that antibody to one serotype of VSV shows CF activity with antigens of the heterologous serotype. The viral antigens responsible for the cross-reacting CF activity were investigated using nucleoprotein core antibody and virus coat antibody.

Cells were infected with the New Jersey serotype of VSV and after harvesting, the virus particles were concentrated and purified by sucrose gradient centrifugation (the same procedures already described for the Indiana serotype were employed). The optical density (280 mμ) along the sucrose gradient was monitored as successive 1 ml fractions were collected. Each fraction of the gradient was separately tested

for CF activity with antibodies to New Jersey virus particles, to Indiana core protein, and to Indiana coat protein.

Fig. 27 a illustrates the CF activity of B particles of the New Jersey serotype (located in fractions 15 to 20 of the gradient). Reactivity to New Jersey virus antiserum was observed as expected in the region of the virus particles. Antibody to core protein of the indiana serotype also showed CF activity with New Jersey virus particles. This result suggests that the core protein of the New Jersey and Indiana serotypes of VSV are antigenically similar. Antibody to Indiana coat protein, on the other hand, failed to show any CF activity with the New Jersey virions. This finding together with the knowledge that coat protein preparations contain the proteins VP_2 and VP_4 suggest that these two proteins are antigenically distinct in the two serotypes. Soluble antigens (20S and 6S components) were also isolated from the New Jersey virus lysates as described in the Methods. In Fig. 27 b, the location of the 20S and 6S antigens in the 5-30% sucrose gradients is apparent from the CF activity with New Jersey virus antibody. The Indiana virus-coat antibody failed to show CF activity with either 20S or 6S New Jersey antigens. Antibody to core proteins of Indiana serotype, however, showed some cross-reacting CF activity with a number of regions in the gradient including that corresponding to the 20S antigen.

These results are consistent with the hypothesis that the 6S soluble antigen of New Jersey virus is, like the

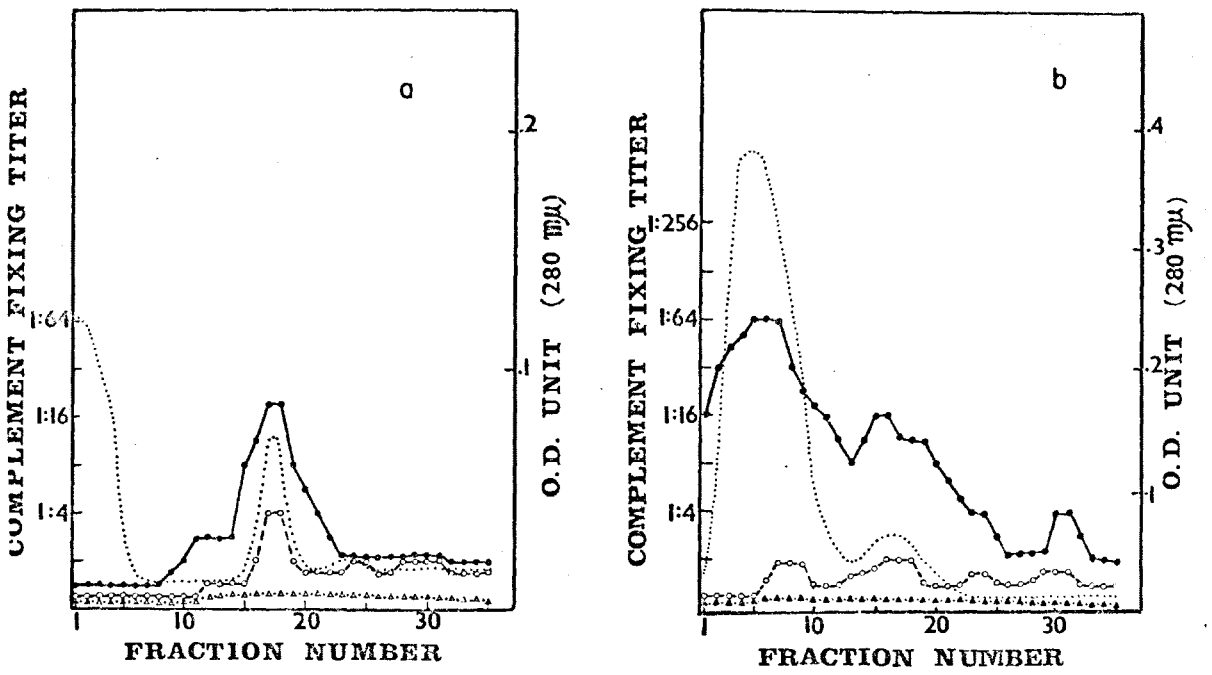


Fig. 27. Complement fixing activity of New Jersey serotype B particles and New Jersey 6S and 20S antigens with New Jersey virus antiserum and heterotypic Indiana core and coat antisera.

New Jersey virus particles and free antigens were purified from infected lysates by differential centrifugation and ammonium sulphate fractionation. The virus was examined by rate zonal centrifugation on 5-30% sucrose gradients at 4°C and 41,000 x g for 60 minutes (a) while 20S and 6S antigens were separated on 5-30% sucrose gradients centrifuged at 4°C and 51,000 x g for 16.5 hours (b). The direction of sedimentation is left to right in the diagrams. The optical density (280 mμ) was monitored continuously along the gradients (.....) as 1 ml fractions were collected. Each fraction was separately tested for CF activity with antiserum to New Jersey virus (●), antiserum to Indiana core antigens (O) and antiserum to Indiana coat antigens (Δ).

coat antigens, distinct from protein produced by the Indiana serotype. The 20S antigen, on the other hand, shows CF cross-reactivity with antibody to ribonucleoproteins, and these proteins are therefore antigenically similar in both serotypes.

15. Virus Neutralization with Specific Antibodies

While cross-reactivity of CF activity occurs between New Jersey and Indiana serotype virus, it was shown by Cotton (1927) that neutralizing antibodies were distinct for each serotype. Concurrent with the results of the previous section, the absence of cross-neutralization of heterotypic antisera (Fig. 28), would strongly suggest that the virus-neutralizing antibody must reside in antibodies directed against components of the virus coat. To check this, a standard amount of HR-LT virus was mixed with serial dilutions of both types of core and coat antibody. After incubation of the mixture at 37°C for 30 minutes, residual viral infectivity was determined by plaque assay. As can be seen in Fig. 29, antibody directed against viral nucleoprotein core had no virus-neutralizing activity. Antibody against virus coat, however, possessed a high degree of neutralizing activity.

The antigenic relatedness of the 6S antigen to a component of the virus coat suggested that it might be possible to remove virus-neutralizing activity from antibody preparations by adsorption with 6S antigen. Accordingly, a constant amount

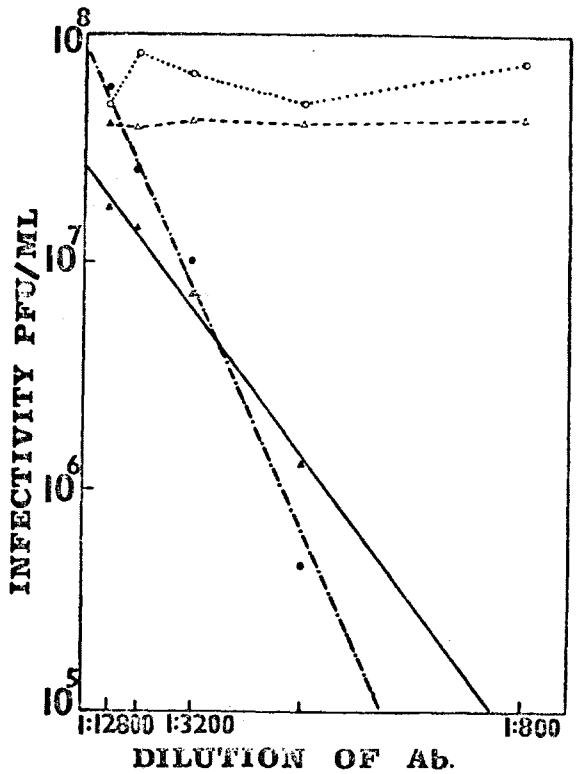


Fig. 28. Absence of cross-neutralization of infectious VSV by heterotypic antisera.
Antisera to partially purified virus particles of the Indiana serotype and of the New Jersey serotype were prepared in rabbits as described in Methods. To 0.5 ml of appropriately diluted antiserum was added an equal volume of either Indiana or New Jersey infectious virus stock. The mixtures were incubated at 37°C for 30 minutes and then appropriately diluted and the residual PFU determined. Antiserum to Indiana virus was tested against infectious Indiana VSV (▲) and New Jersey VSV (o) and antiserum to New Jersey virus was also tested against Indiana VSV (△) and New Jersey VSV (●).

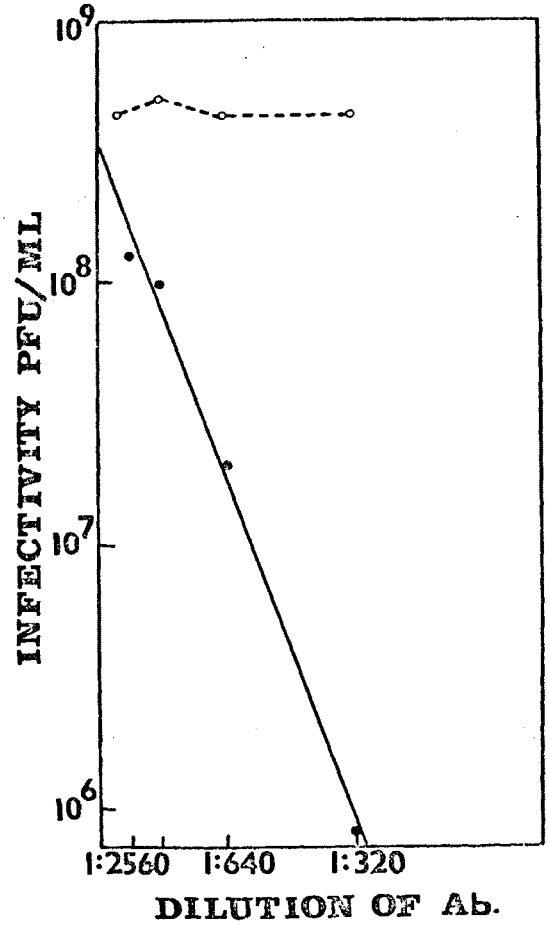


Fig. 29. Virus neutralization using antibody to viral nucleoprotein core and antibody to viral coat. To serial dilutions of either Indiana core Ab (o) or Indiana coat antibody (●) was added an equal volume (0.5 ml) of stock Indiana VSV containing 5 x 10^8 PFU. The mixtures were incubated at 37°C for 30 minutes, at which time aliquots were removed, appropriately diluted and the residual PFU determined.

of the antibody was mixed with serial fourfold dilutions of 6S antigen, and the mixtures were incubated at 37°C for 6 hours. The residual neutralizing activity of antiserum was tested by adding 2×10^7 plaque forming unit per 0.1 ml of VSV to each reaction mixture. After incubation at 37°C for 30 minutes, the residual infectivity in each tube was checked by plaque assay. As indicated in Table 2, 6S antigen completely blocked the neutralizing activity of the virus antiserum.

This observation not only confirms the relatedness of the 6S antigen to an antigen component of the virus coat, but indeed suggests that neutralizing antibody to the virus may be directed against the glycoprotein VP_2. This conclusion would confirm that the observation made by Cartwright et al. (1970) that VP_2 constitutes the spikes projecting from the viral surface since it might be expected that neutralizing antibody would be directed against surface components of the virus.

16. Immune Precipitation of the 6S Antigen

With the availability of antiserum capable of reacting with the 6S antigen, it was possible to establish beyond doubt that the radioactivity present in the 6S antigen region of lysates grown in ^{14}C-amino acids is indeed in virus-specific protein and not in co-sedimenting cellular proteins.

Table 2

Serum Blocking Power of 6S Antigen

Serum Blocking	
Dilution of 6S Antigen	Surviving Infectivity PFU/ml
Undiluted	2.3×10^7
1/4	1.5×10^7
1/16	3.1×10^5
1/64	4.4×10^3
1/256	3.4×10^2
1/1024	2.1×10^2
Ab without 6S Ag	1.4×10^2
Control virus titer	2.1×10^7

Sucrose gradient purified 6S antigen was prepared from Indiana VSV lysates. Serial four-fold dilutions of 6S antigen (0.5 ml each) were prepared. To each antigen dilution was added 0.5 ml of a standard dilution of anti-Indiana VSV antiserum. The antigen-antiserum mixtures were incubated at 37°C for 6 hours. At this time each mixture received 2×10^7 PFU of Indiana VSV in 0.1 ml. Incubation was continued at 37°C for 30 minutes at which time the mixture was appropriately diluted and the residual viral infectivity determined (PFU/ml). Controls showing the virus titer after the addition of antiserum alone and the virus titer in the absence of antiserum are included.

For this purpose, ^{14}C-labelled 6S antigen was prepared and purified by methods previously described. Equal quantities of radioactive 6S antigen were mixed with 10 volumes of serial two-fold diluted Indiana whole virus antibody. The mixtures were incubated at 37°C for 1 hour and then were stored at 4°C for another 48 hours. The immune precipitates were collected by centrifugation, and radioactivity remaining in the supernatant fluid was determined (Table 3).

The immune precipitates were pooled, and, after treatment with urea, SDS and mercaptoethanol, were subjected to polyacrylamide gel analysis. The results (Fig. 30) demonstrated that VP_{2a} is indeed a virus-specific antigen and not a cellular protein.

Recently, a number of papers, some seemingly conflicting, have described the intracellular proteins of VSV infection (Wagner et al., 1970; Petric and Prevec, 1970; Burge and Huang, 1970; Mudd and Summers, 1970). While both Petric and Prevec, Burge and Huang reported the presence of only four virus-specific intracellular proteins identical to the four virion proteins (Fig. 15); Wagner et al. (1970) have suggested that two additional non-virion, virus-specific proteins, designated NS1 and NS2, are present in infected cells. Mudd and Summers (1970) have reported detecting some five to ten proteins within virus-infected HeLa cells. These latter workers have also

Table 3.

^{14}C-labelled 6S antigen was prepared and purified from Indiana VSV infected cells. A parallel fraction from ^{14}C-labelled but noninfected cell cultures was also obtained. Serial two-fold dilutions of Indiana whole virus antiserum were prepared (0.5 ml each) and to each was added an equal volume of either infected or uninfected 6S material. A sample, 0.05 ml in volume, was immediately removed from each tube and the radioactivity determined. The tubes were incubated at 37°C for 1 hour and then stored at 4°C for a further 48 hours. The precipitate was then removed by centrifugation at 1500 x g for 10 minutes and 0.05 ml removed from the supernatant and its radioactivity determined. The difference between the radioactivity in the initial sample and that remaining in the supernatant after immune precipitation is expressed as percent of the initial radioactivity in the third column.

Table 3

Immune Precipitation of 6S Antigen

Dilution of Ab.	6S Fraction from Infected Culture			6S Fraction from Noninfected Culture		
	cpm/0.5 ml total mixture	cpm/0.5 ml remaining in supernatant	% of total cpm in immune precipitate	cpm/0.5 ml total mixture	cpm/0.5 ml remaining in supernatant	% of total cpm in immune precipitate
1/2	1350	810	40.0	880	890	0
1/4	1234	827	33.1	827	798	3
1/8	1256	885	29.6	821	802	2
1/16	1081	926	14.3	855	860	0
1/32	1140	1034	9.3	800	795	0
1/64	1191	1092	8.3	783	803	0
1/128	1130	1083	5.1	790	795	0
1/256	1132	1104	2.3	781	780	0
1/512	1060	1085	0	770	750	3
1/1024	1070	1074	0	720	733	0
No Ab.	1050	1055	0	790	785	0

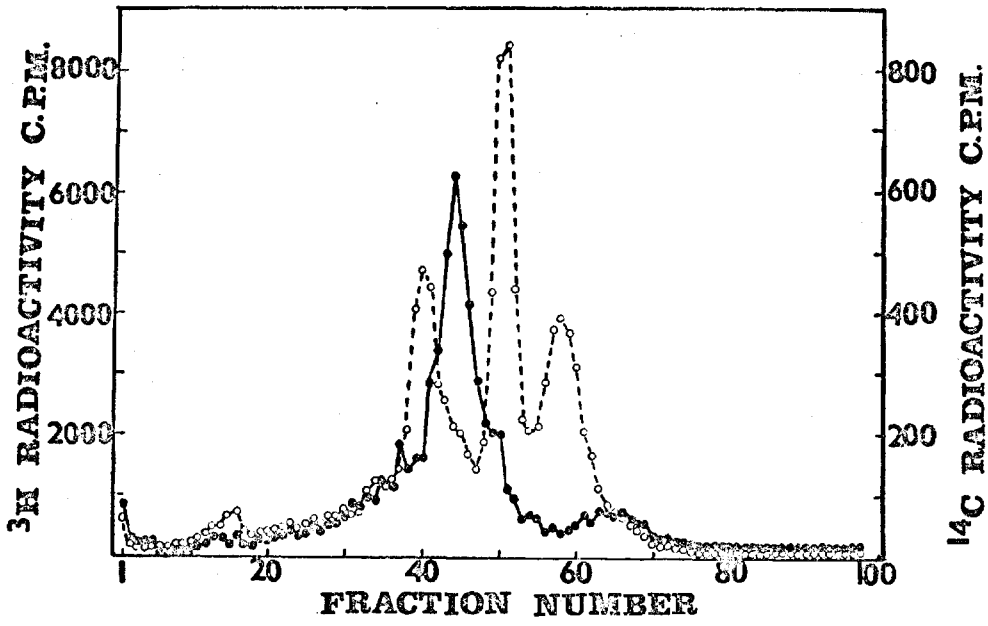


Fig. 30. Polyacrylamide gel analysis of proteins in immune precipitate of 6S antigens.
The ^{14}C-labelled protein of the immune precipitate described in Table 3 was washed with ethanol, mixed with marker ^{3}H-labelled viral structural protein, denatured and analyzed on 5% polyacrylamide gels. The ^{3}H-radioactivity (o) and ^{14}C-radioactivity (●) was determined as described in Fig.

observed an additional (fifth) protein in the virion.

The subsequent experiments illustrate the intracellular and extracellular proteins present during VSV infection and suggests what appears to be a consistent picture of virus directed protein synthesis.

17. Virus-Specific Intracellular Proteins

Infection of cells with VSV at high multiplicities of infection results in an inhibition of cellular protein synthesis (Wagner et al., 1970). In order to determine if all remaining protein synthesis is in fact virus specific, cells were treated with Actinomycin D (Act D) for 20 hours prior to high multiplicity infection. The polyacrylamide gel electrophoretic profiles of intracellular proteins made between one and two and one-half hours post-infection in cells with or without Act D pretreatment is shown in Figs. 31 a and 31 b respectively.

A comparison of these figures shows that pretreatment of cells with Act D was effective in reducing the background level of protein synthesis below that observed with infection alone. Act D treatment resulted in less than two-fold reduction of virus yield compared to untreated cells. Extending the pretreatment to 36 hours did not substantially improve the result.

It should be noticed in Fig. 31 b that the position in the gel of the virion marker protein VP_2 does not

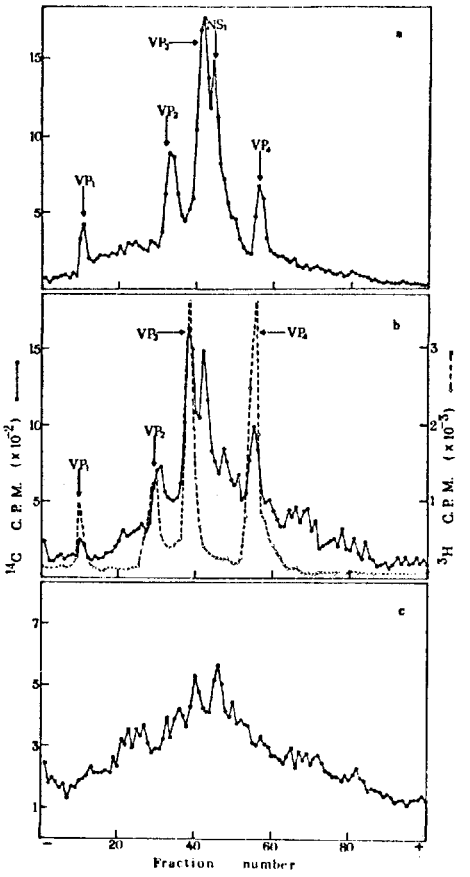


Fig. 31. Radioactive proteins in infected cultures with and without Act D pretreatment and in non-infected cultures after Act D pretreatment.
Some 10^8 cells were treated for 20 hours with Act D as described in Methods and then infected with 100 PFU/cell of infectious VSV stock. Radioactive ^{14}C-amino acids were added at one hour post-infection and the culture harvested at two and one-half hours post-infection. The radioactive proteins in the cellular fraction were prepared as described in Methods and analyzed on 7.5% polyacrylamide gels. The resultant distribution of radioactivity along the gel is shown in a (●). The results of an identical experiment omitting the Act D pretreatment is shown in b (●). The dotted curve (○) shown in b represents the ^3H radioactivity of marker proteins from purified virions. The radioactive proteins from cells pretreated for 20 hours with Act D and labeled for 1.5 hours with ^{14}C-amino acids in the absence of infection are shown in c (●).

correspond with the position of the intracellular VP_2. This protein in the cell always migrates slightly faster than the virion protein but not as fast as the soluble glycoprotein VP_{2a}. This observation, along with relevant later results, will be considered in the discussion.

Fig. 31 a shows that, despite the pretreatment with Act D, an additional protein distinct from the virion proteins is present in the infected cells. This protein is designated NS1 following the nomenclature of Wagner et al. (1970). While Fig. 31 b suggests the presence of other non-virion proteins, most of these were eliminated by Act D pretreatment. Even with pretreatment however, some radioactive protein is observed in the position between NS1 and VP_4.

While this material was seen in all experiments, the lack of sharp peaks and lack of consistency in position suggests that this represents residual protein synthesis which has not been eliminated by either Act D pretreatment or infection. Fig. 31 c shows that uninfected cultures, pretreated for 20 hours with Act D, still show significant residual synthesis of protein species which migrate to this region of the gel.

In order to confirm that NS1 is a virus-specific protein, cultures of primary chick embryo, human KB and human amnion (FL) cells were pretreated with Act D, infected and labelled exactly as in the above experiment. In all cases the protein NS1 was present in about the same ratio to VP_3

as it was in L-cells.

From these experiments it is concluded that NS1 is a virus-coded protein.

18. Continuous Labelling of Virus-Specific Proteins

The kinetics of virus protein synthesis was investigated by three distinct experiments. The first of these examined viral protein synthesis during continuous exposure to radioactive amino acids.

A suspension culture of L-cells, pretreated for 20 hours with Act D, was infected with VSV at a moi of 200 and 0.5 μc/ml of ^{14}C-amino acids added at 2 hours post-infection. Aliquots containing 3 x 10^7 cells were removed at 3, 4, 5, 6 and 7 hours post-infection and after removing a portion for plaque assay and determination of acid precipitable radioactivity, the material in each aliquot was fractionated into cells, virions, and soluble proteins by differential centrifugation and ammonium sulphate precipitation as described in Methods. The accumulation with time of acid insoluble radioactivity in the total culture, in virions and in the soluble protein fraction is presented in Fig. 32. It can be seen that the rate of protein synthesis in the total culture increased linearly to 5 hours post-infection and then decreased significantly. Radioactive protein continued to be released to the virion, and soluble fractions at a rapid rate up to 7 hours post-infection. It

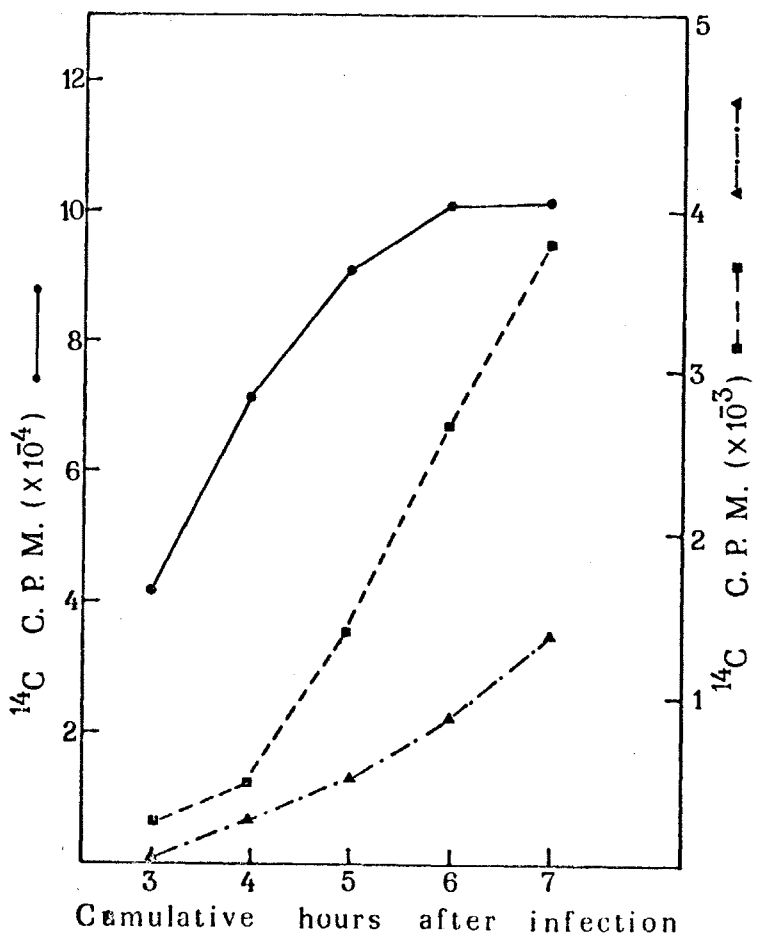


Fig. 32. Cumulative labelling of virus specific protein following infection.
Approximately 1.5 x 10^8 cells were pretreated with Act D for 20 hours, infected at an moi of 200 PFU/cell and ^{14}C-amino acids added at two hours post-infection all as described in Methods. One hour later and at each hour until seven hours post-infection, one-fifth of the infected culture was removed. One ml of this sample was precipitated with cold TCA to allow computation of the total radioactivity (O). The remaining portion of the sample was used to prepare cellular, virion and soluble antigen fractions as described in Methods. The total radioactivity in the virion (■) and soluble antigen (▲) fractions are shown for each time point (note the factor of 10 difference in the radioactivity scales).

should be noted that even at this late time over 90% of the total radioactive protein in the culture was cell associated, though virtually all of the infectious virus was in the cell free supernatant.

Examination of the viral proteins in the cells, virions or soluble protein fractions by polyacrylamide gel electrophoresis failed to reveal any major changes with time of labelling. Continuous labelling procedures lack the sensitivity to reveal the changes in protein synthesis and maturation of the virion is revealed by subsequent experiments.

19. Pulse-Labelling of Virus-Specific Proteins

This kinetic experiment was done a) to determine if the decrease in rate of protein synthesis at late times was real or due only to the exhaustion of labelled precursors, and b) to determine the rate of synthesis and rate of extracellular appearance, after infection, of the virus specific proteins.

Cells were pretreated with Act D and infected exactly as had been described for the previous experiment. At various times post-infection an aliquot of 5×10^7 infected cells was removed from the culture, the cells collected by centrifugation and resuspended in pre-warmed amino acid-deficient medium containing ^{14}C-amino acids mixture. After 1.5 hours incubation a small sample was removed for plaque assay and determination of TCA precipitable

radioactivity and the remaining sample was fractionated into infected cells, virions and soluble proteins.

As seen in Fig. 33, the rate of protein synthesis was maximal between 2.5 to 4.0 hours post-infection, decreasing after this time in agreement with the previous experiment. It is also evident that the rate of incorporation of synthesized viral proteins into virion (i.e., release of labelled protein as virus or soluble antigen) remained relatively constant throughout the infectious cycle, about 10% of the label being extracellular after any 1.5 hour labelling period.

When the individual labelled proteins of each fraction were examined by polyacrylamide gel electrophoresis it was possible to determine the amount of synthesis of each protein species over the 1.5 hour periods. For this calculation protein VP_3, arising from the 20S soluble antigen, was combined with both virion and intracellular VP_3 while the fraction called VP_2 includes, as well as virion VP_2, the 6S antigen (glycoprotein VP_{2a}) and the intracellular VP_2. More will be said of these proteins in the next section. Fig. 34 a shows the absolute amount of radioactivity incorporated into each protein species in any 1.5 hour period.

Fig. 34 b shows the same data plotted to represent the amount of each protein species as a percent of the total synthesized protein in that time interval. From this curve, it can be seen that while VP_3 and VP_1 form a relatively

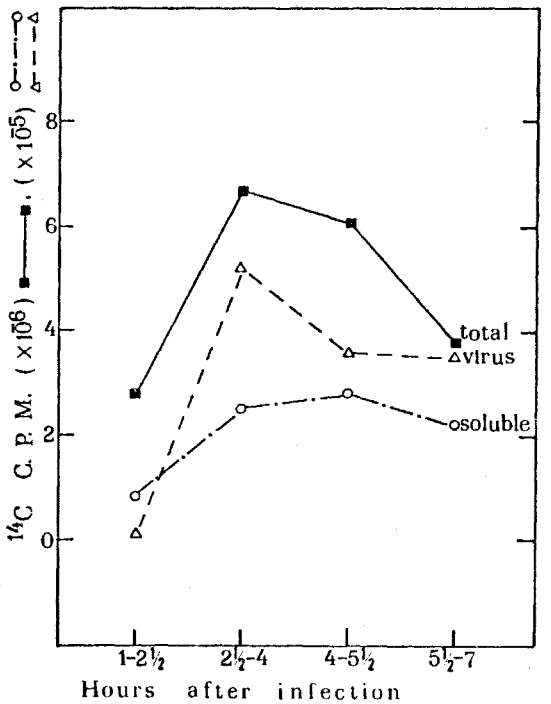


Fig. 33. Pulse-labelling of infected cell cultures at various times post-infection.
Cells were pretreated with Act D and infected as described for Fig. 32. At 1, 2.5, 4 and 5.5 hours post-infection some 5×10^7 cells were removed from culture and incubated with ^{14}C-amino acids for 15 hours. At the end of this labelling period 1 ml of the culture was acidified with TCA and the total radioactivity computed (■). The remaining sample was fractionated into cellular, virion and soluble protein fractions as described in Methods and the total radioactivity in the virion (Δ) and soluble protein (O) fractions determined. Note the change in scales by a factor of 10.

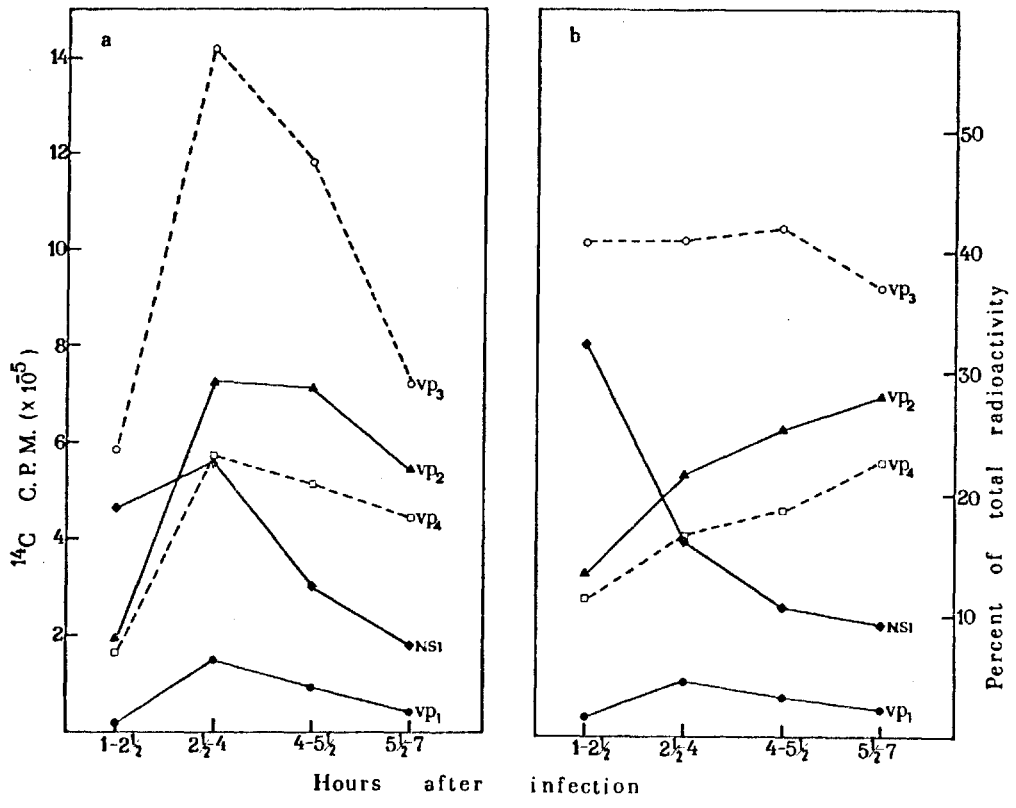


Fig. 34. Rate of synthesis of each virus-specific protein at different times post-infection.
The proteins of the cellular, virion and soluble protein fractions derived from the experiment described in Fig. 33 were analyzed by polyacrylamide gel electrophoresis. The relative proportion of each of the five major virus-specific proteins was determined at the absolute amount of radioactivity in each protein of that fraction computed as described in Methods. The total amount of each protein produced during the 1.5 hour labelling period was obtained by addition as discussed in Results and plotted in a. This same data expressed as a percentage of the total protein synthesized in any time interval is presented in b.

constant proportion of the virus protein synthesized at all times, the proportion of VP_2 and VP_4 synthesized increases with time of infection while that of NS1 decreases considerably at later times.

This experiment demonstrates the independent synthesis of virion proteins and suggests that a temporal control of protein synthesis may be operative during the VSV infection cycle.

20. Pulse-Chase of Virus Specific Proteins

For this final kinetic experiment cells were pretreated with Act D and infected exactly as described above. At 2.5 hours post-infection the cells were collected by centrifugation and resuspended in low amino acid medium containing ^{14}C-amino acids. After 30 minutes labelling, the cells were collected by centrifugation and resuspended in normal MEM containing unlabelled amino acids. One sample was removed from the culture immediately after resuspension and one at each of the next three subsequent hours.

Each sample was fractionated into cells, virions and soluble proteins after determination of total radioactivity. As seen in Fig. 35 the total radioactive protein in the culture remained constant showing that the chase had been effective in preventing continued incorporation of label. Under these conditions, the intracellular radioactivity decreased continuously while that of the virion and soluble

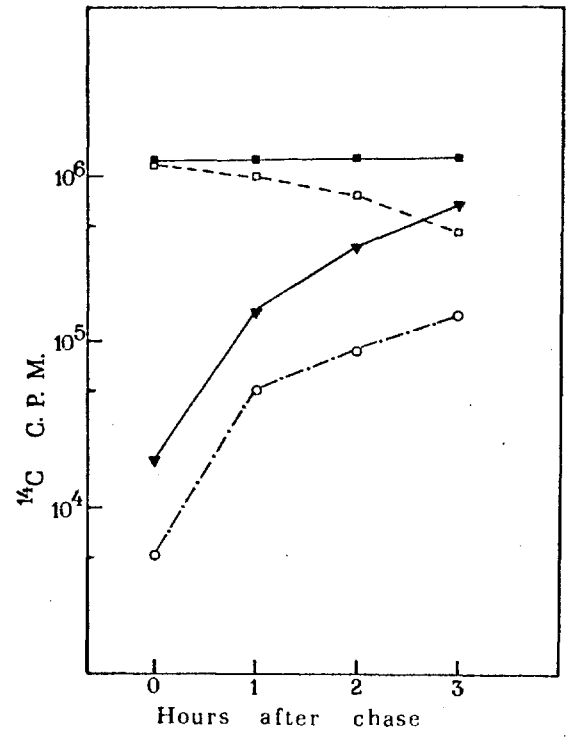


Fig. 35. Movement of radioactive virus-specific proteins from intracellular to extracellular location during a pulse-chase experiment.
Cells were pretreated with Act D and infected as described in Fig. 32. ^{14}C-amino acids were added to the culture at 2.5 hours post-infection and then removed 30 minutes later by centrifugation and resuspension of the infected cells in normal non-radioactive MEM. The zero time sample was removed from the culture at this point and subsequent samples were taken at hourly intervals. The radioactive protein in 1 ml of the sample was collected by TCA precipitation and the total acid precipitable radioactivity in the culture determined and plotted (■). The material in each sample was fractionated into cellular, virion and soluble protein fractions as described in Methods. The total acid precipitable radioactivity at each time point for the cellular fraction (□), virion fraction (▽) and soluble protein fraction (o) are plotted.

proteins constantly increased showing that maturation of virion continued during the chase period.

The polyacrylamide gel patterns of radioactive proteins from the cells, virions, and soluble protein fractions for each time point are shown in Figs. 36, 37 and 38 respectively. The absolute radioactivity in each of the intracellular proteins is summarized in Fig. 39 a. From this result it can be seen that under these conditions of chase the intracellular half lives of proteins VP_1, VP_2 and VP_3 are approximately 2 hours, that of VP_4 is about one hour, and that of NS1 is greater than five hours. This result is reflected in the percentage of the total intracellular protein accommodated by each respective viral protein, as seen in Fig. 39 b. Thus, while VP_1, VP_2 and VP_3 remain a fairly constant percentage of the total, VP_4 is a decreasing and NS1 an increasing percentage of the total radioactive intracellular protein during the chase.

This result suggests that the protein NS1 may have primarily an intracellular function.

In a similar way, Fig. 40 a shows the accumulation of radioactivity in the extracellular protein during the chase. In this graph the 6S antigen (VP_{2a}) was considered both alone and together with virion protein VP_2 and the 20S antigen (VP_3) was plotted as both 20S antigen with virion VP_3 and virion VP_3 alone.

As demonstrated in Fig. 40 b though radioactivity

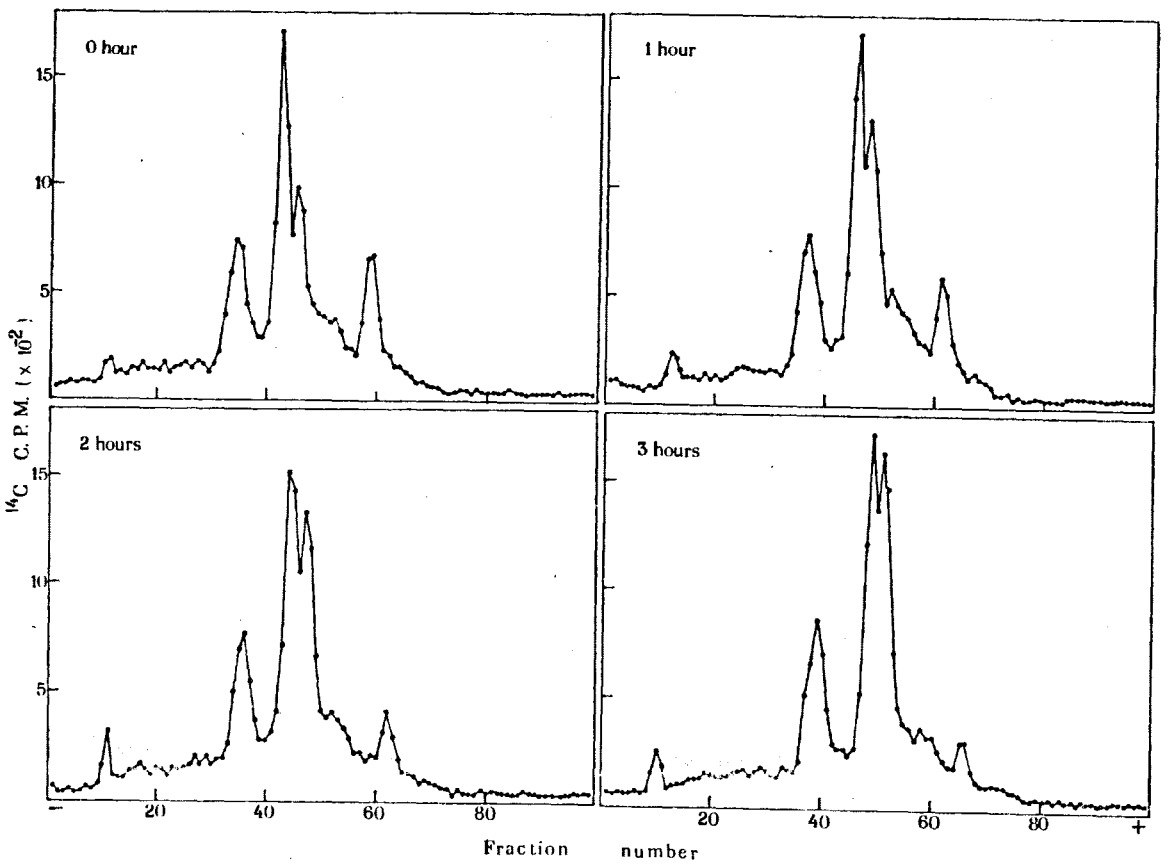


Fig. 36. Polyacrylamide gel electrophoresis patterns of radioactive virus-specific proteins in the cellular fraction during a pulse-chase experiment.
The cellular fraction taken from the experiment described in Fig. 35 was disrupted and approximately 150,00 cpm of radioactive protein analyzed on 7.5% polyacrylamide gel.

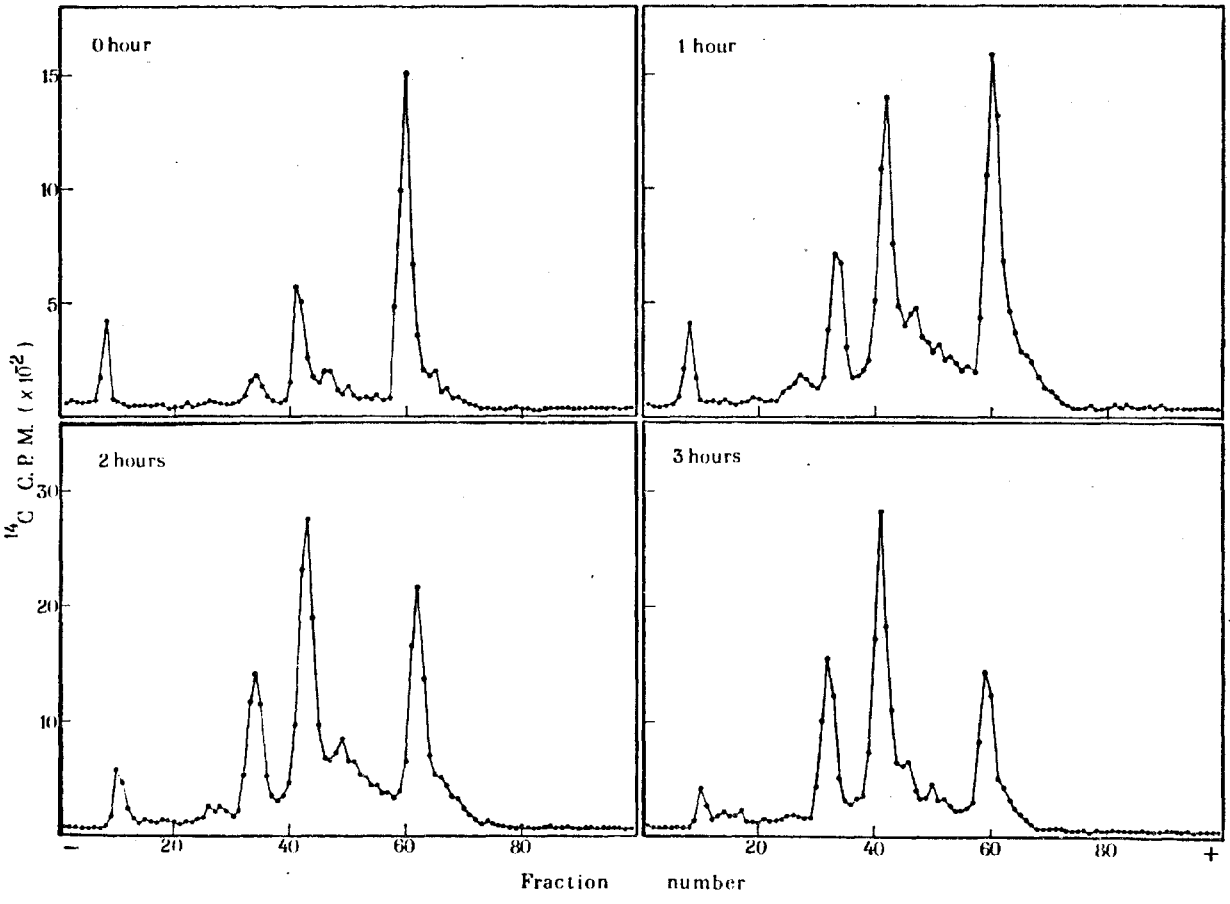


Fig. 37. Polyacrylamide gel electrophoresis of radioactive virus-specific proteins appearing in the virion fraction during a pulse-chase experiment.
The virion fractions taken from the experiment described in Fig. 35 were disrupted and up to 200,000 cpm of the radioactive protein analyzed on 7.5% polyacrylamide gels.

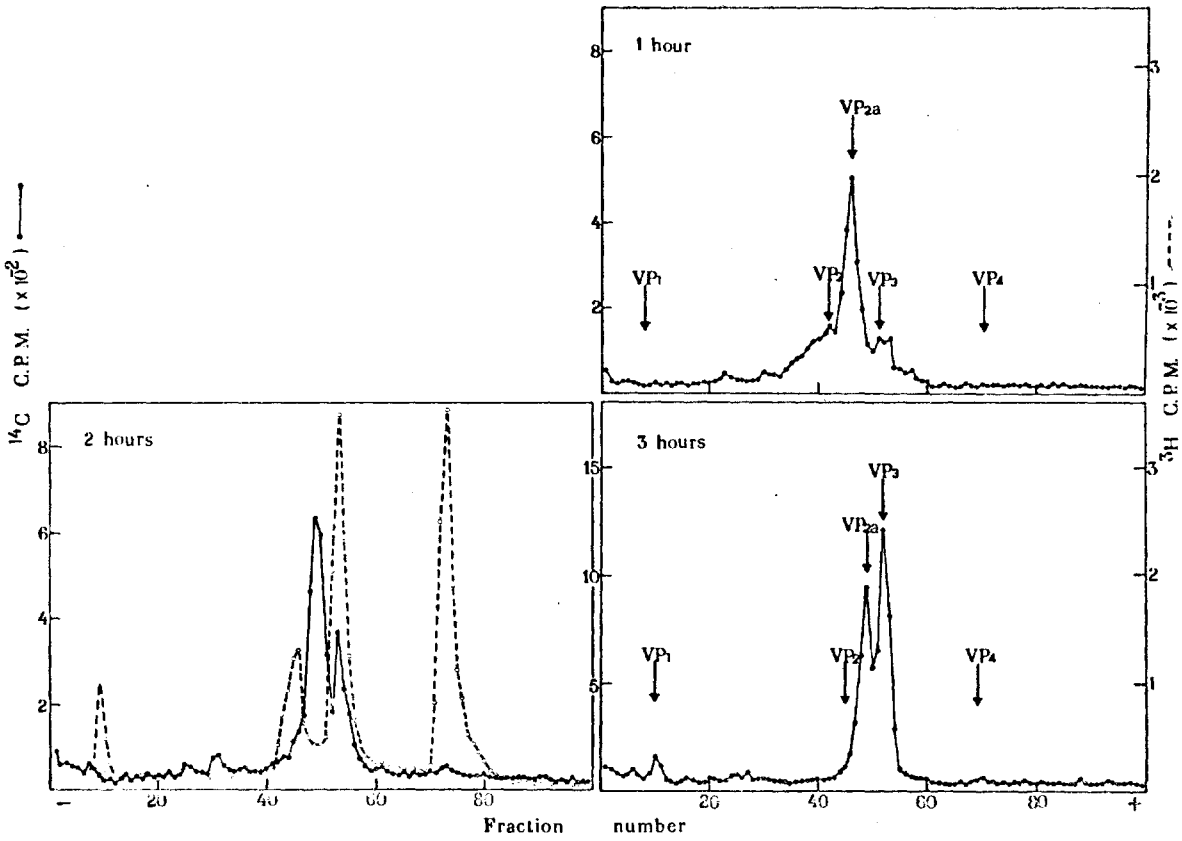


Fig. 38. Polyacrylamide gel electrophoresis of radioactive virus-specific proteins appearing in the soluble protein fraction during a pulse-chase experiment.
The soluble protein fractions taken from the experiment described in Fig. 35 were disrupted and approximately 20,000 to 30,000 cpm of ^{14}C radioactive protein mixed with approximately 100,000 cpm of ^{3}H virion marker was analyzed on 7.5% polyacrylamide gels. There was insufficient radioactive protein in the soluble protein fraction at the zero time of chase to allow analysis in this manner. Although the ^{3}H-marker protein (o) is plotted in only one case the location of the virion proteins is indicated by arrows in the other diagrams.

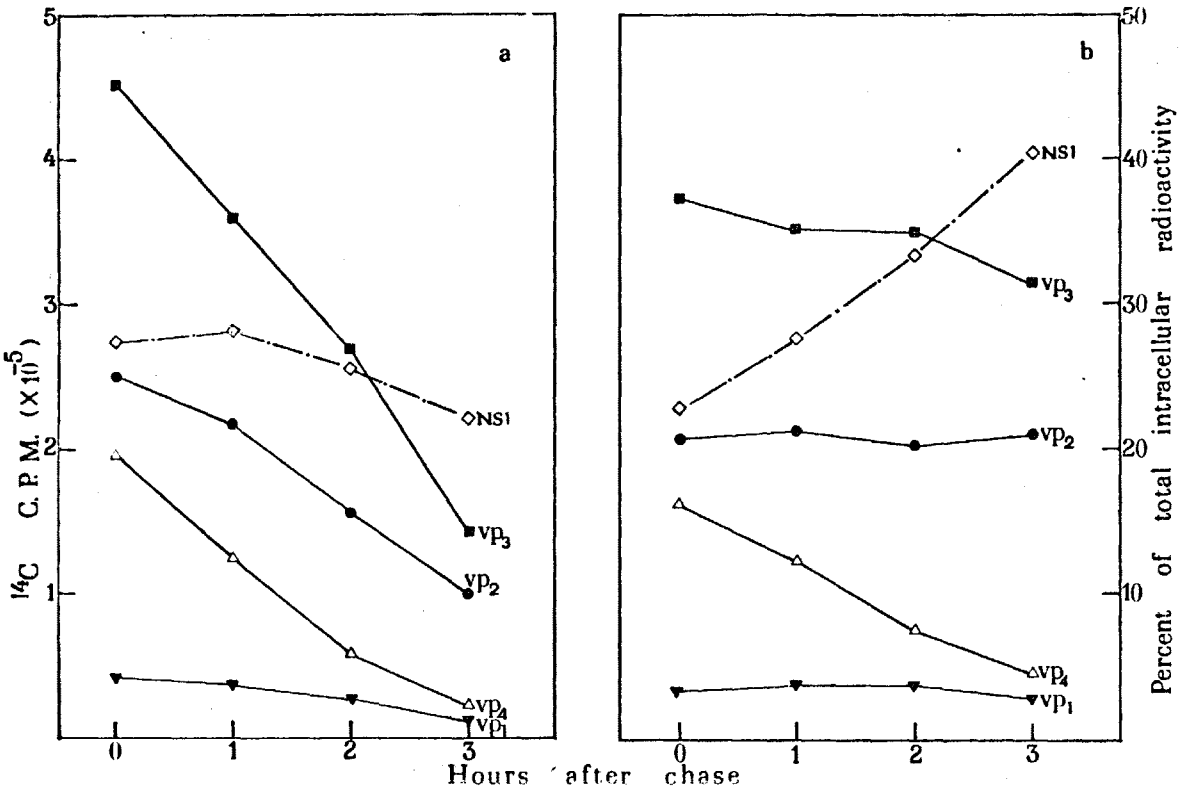


Fig. 39. Movement of individual radioactive virus-specific proteins from the infected cell following a short pulse. Knowing the total radioactive protein in the cell at hourly intervals following a 30 minute radioactive pulse (Fig. 35) and knowing the relative ratios of each of the virus-specific proteins present at each time (Fig. 36) allowed a calculation of the total radioactivity in each protein in the infected cell during the chase period (a). The same data shown in a were recomputed so as to express the amount of each protein present in the cell as a percentage of the total radioactive protein in the cell at that time (b).

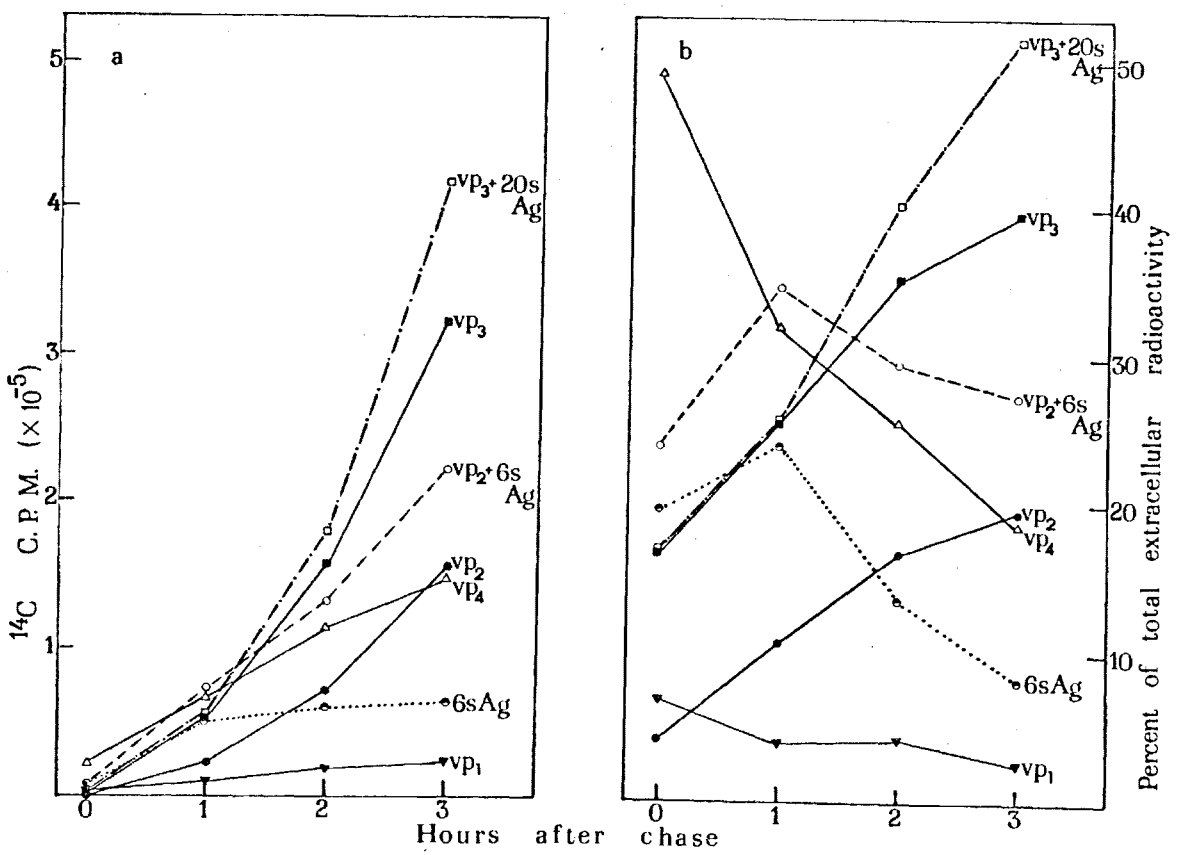


Fig. 40. The appearance in the cell free supernatant of individual virus-specific proteins following a short pulse. From the data presented in Fig. 35 and that in Figs. 37 and 38, it was possible to compute the total amount of radioactivity in each virus-specific protein present in the extracellular fluid after a 30 minute pulse of radioactivity. The result for all of the distinguishable protein fractions is plotted in a. The same data shown in a were recomputed so as to express the amount of each extracellular protein as a percentage of the total extracellular radioactivity at that time (b).

accumulates in all extracellular proteins, the rate of appearance in VP_2 and VP_3 increases during the chase while the rate of appearance of radioactive VP_1, VP_4 and 6S antigen decreases.

This result suggests that the proteins VP_3 and VP_2 undergo some assembly within the cell prior to release whereas both VP_1 and VP_4 are released soon after synthesis.

21. Intracellular Ribonucleoprotein Complex in VSV Infected Cells

Throughout these kinetic experiments, VP_1 is considered as a distinct protein species; however, both Mudd and Summer (1970) and Wagner et al. (1970) have suggested, without experimental evidence, that it may be an artifact of protein aggregation. Accordingly, an attempt was made to determine the intracellular location and hence the possible function of VP_1 in infected cells.

Suspension L-cell cultures were pretreated with Act D and infected at an moi of 50 with plaque purified B particles as stock virus. The infected cells were labelled with a ^{14}C-amino acid mixture between 2.5 to 5 hours post-infection and cytoplasmic extracts prepared as described in Methods.

The cytoplasmic extract was then analyzed on a sucrose gradient. The resultant gradients were collected with continuous monitoring of optical density.

Fig. 41 a shows the polyribosome profile with the

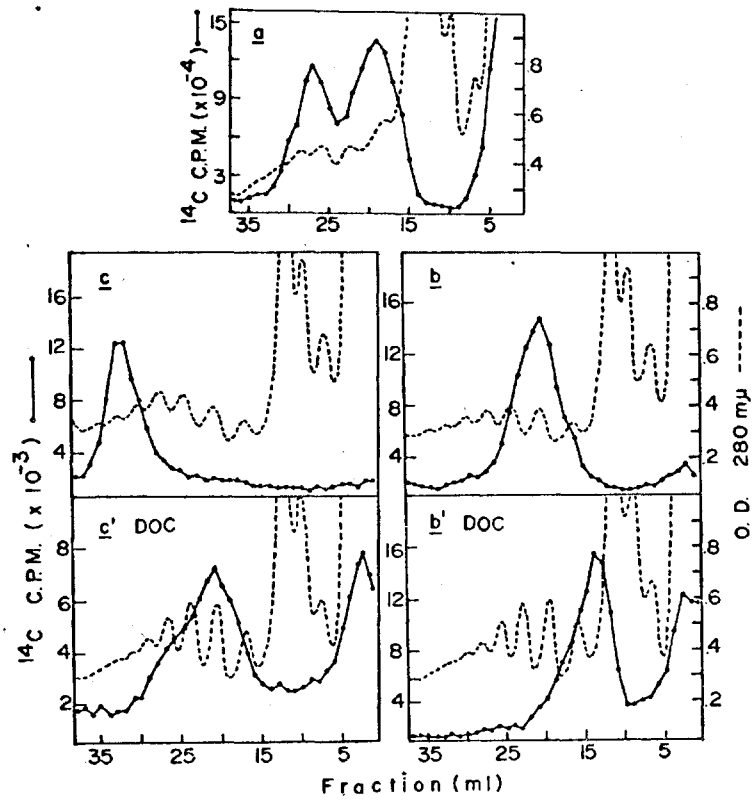


Fig. 41. Sucrose gradient analysis of ^{14}C-amino acid labelled cytoplasmic structures obtained from VSV infected culture.
Approximately 10^8 cells were pretreated with 0.5 μgm/ml of Act D for 12 hours, then infected with pure B particles at 100 PFU/cell and labelled with ^{14}C-amino acid mixture between 2.5 and 5 hours post-infection. The harvested cells were washed in PBS and resuspended in RSB°. The cells were homogenized in a glass Dounce homogenizer and the nuclear and cell membrane fractions removed. The resultant cytoplasmic fraction was then layered on the top of a 15-30% sucrose gradient made in RSB° and centrifuged for 3.5 hours at 24,000 rpm, 5°C in a Spinco SW27 rotor. The gradients were collected with continuous monitoring of optical density and successive 1 ml fractions collected. A portion of each fraction was placed directly in 5 ml of Bray's solution and radioactivity determined (a). The peak at fraction number 18 to 20 and the peak at fraction number 26 to 28 were pooled separately and dialyzed in RSB° at 4°C overnight. Both radioactive peaks were split into two portions; one portion was applied directly to the top of a 15-30% sucrose gradient in addition to uninfected cell cytoplasmic extract as marker (b and c) while the second portion was made in 0.5% DOC and then placed on a second gradient (b' and c') and analyzed as in (a).

distribution of virus specific radioactive proteins in the cytoplasmic fraction. As can be seen, the cytoplasmic extract contains virus specific proteins in the slowly sedimenting material at the top of the gradient and in fractions which sediment further down the gradient in the region of approximately 140S and 160S.

These 140S and 160S peaks were pooled separately and dialyzed in RSB° at 4°C overnight.

The samples were split into two identical portions, each one of which sample was then treated with DOC and the sedimentation velocity profiles re-examined on sucrose gradients in the presence of uninfected L-cell cytoplasmic extract as a marker. The untreated samples again show major peaks of radioactivity at 140S (Fig. 41 b) and 160S region (Fig. 41 c), and so the sedimentation velocity property did not change appreciably. Treatment with DOC however resulted in the disappearance of the 140S peak and its replacement by a peak with sedimentation coefficient of approximately 100S (Fig. 41 b'). The 160S peak also disappears after DOC treatment and is replaced by a more heterogeneous peak with sedimentation coefficient of 140S (Fig. 41 c'). This new 140S peak, derived from 160S by DOC treatment, will be denoted as DOC-140S in order to distinguish it from the 140S peak present in the untreated sample.

It would thus appear that material sedimenting at 140S and 160S are converted by DOC to material with

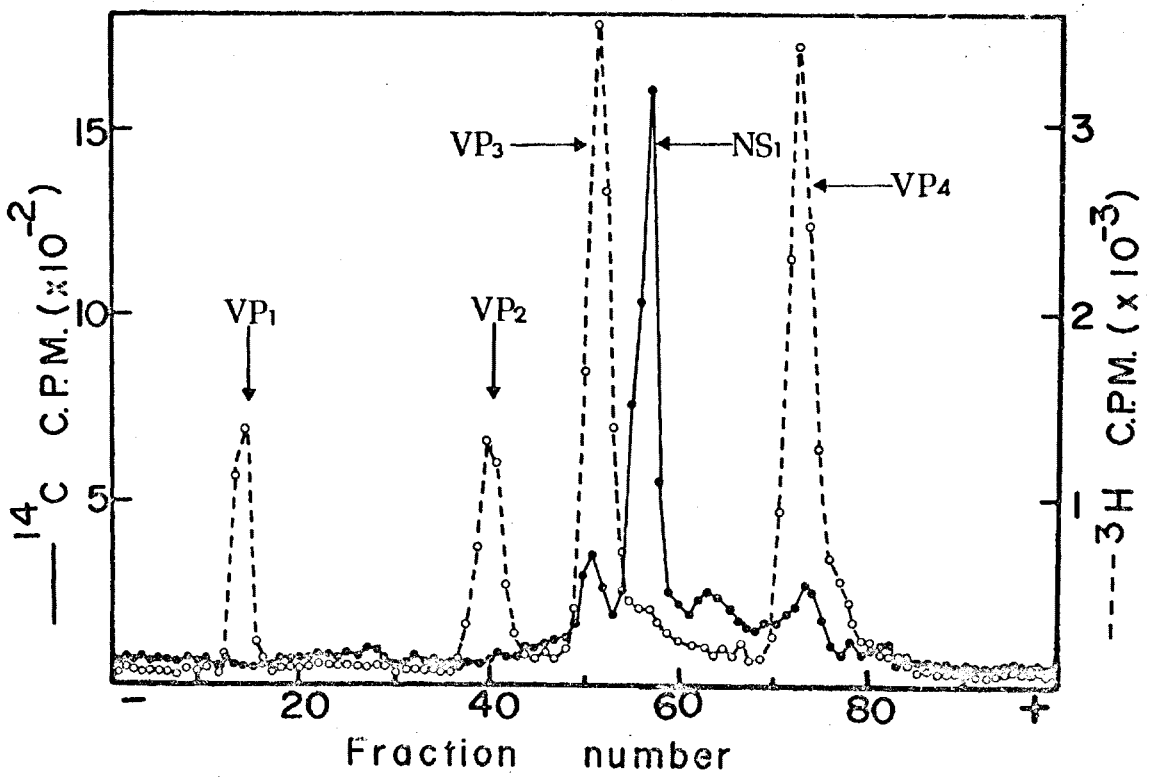


Fig. 42. Polyacrylamide gel profile of ^{14}C-radioactive protein fraction taken from cytoplasmic extract of VSV infected cells.
Radioactive proteins were precipitated with 95% ethanol from fractions 2 and 3 of the sucrose gradient shown in Fig. 41 a. The proteins were analyzed on a 7.5% polyacrylamide gel as described in the Methods. The gels were sliced, the slices depolymerized with hydrogen peroxide, and radioactivity was determined. The ^{3}H markers locate the position of structural proteins obtained from purified virus.

sedimentation coefficients of 100S and DOC-140S respectively. The DOC-mediated lowering of sedimentation coefficient was accompanied by the release of proteins to the top of the sucrose gradients in both cases. It was of some interest therefore to examine the protein constituents present in each of the gradient fractions both before and after DOC treatment.

First of all it can be seen in Fig. 42 that the material at the top of the original cytoplasmic extract consisted almost entirely of NS1. This is in agreement with the observation of Wagner et al. (1970) who showed this protein to be present in the soluble fraction of the cytoplasm.

On the other hand, the original 140S and 160S components both contain proteins VP_1, VP_3 and NS1 as seen in Fig. 43 a and 43 d respectively. DOC treatment of both of these fractions produces the altered protein profiles seen in Fig. 43 b and 43 e with only VP_3 and NS1 remaining; the material at the top of the gradient contains VP_1 and some VP_3 (Fig. 43 c and 43 f).

It would seem, therefore, that conversion of the 140S and 160S material to 100S and DOC-140S by DOC is accompanied by the release of all VP_1 and some VP_3 from these fractions.

This result strongly suggests that VP_1, rather than being an artifact of protein aggregation, may indeed be a functional virus-specific unit.

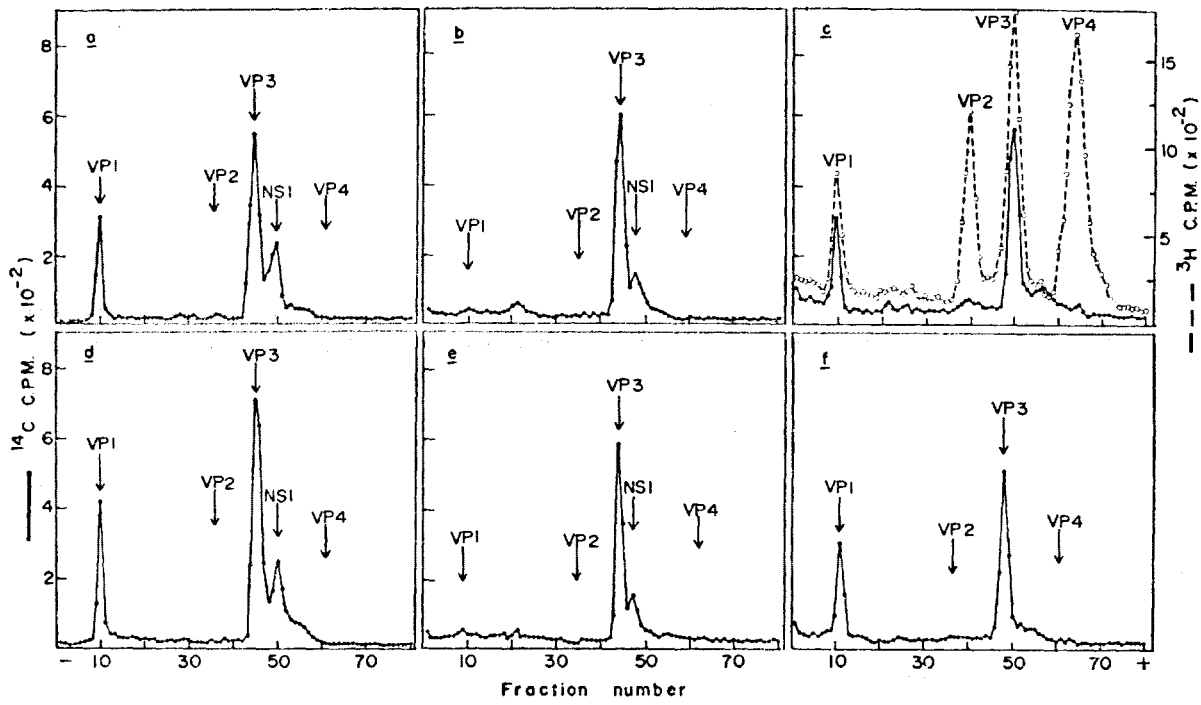


Fig. 43. Polyacrylamide gel profiles of ^{14}C-radioactive protein fractions taken from cytoplasmic extracts of VSV infected cells.

Radioactive proteins were precipitated with ethanol from selected fractions of the sucrose gradients shown in Fig. 41 and the proteins were analyzed on 7.5% polyacrylamide gel. The gels were sliced, the slices depolymerized with hydrogen peroxide at 50°C in a water-saturated incubator for 4 hours, and radioactivity was determined. The material shown in a was obtained from 140S region of the gradient shown in Fig. 41 b (fraction 19 to 22). The material in b was taken from 100S region of the gradient shown in Fig. 41 b' while that in c was taken from the top (fraction 1 to 5) of the same gradient. The result shown in d was obtained from protein taken from the 160S region of the gradient shown in Fig. 41 c (fractions 30 to 34). The result in e was obtained from DOC-140S from the gradient shown in Fig. 41 c' (fractions 19 to 23) while that in f was taken from the top (fractions 1 to 5) of the same gradient. The ^{3}H radioactivity presented in c locates the position of marker structural proteins obtained from ^{3}H labelled purified virion. All the other samples were also co-electrophoresed with ^{3}H structural protein markers and the position of the proteins are indicated with arrow marks.

22. Preparative Polyacrylamide Gel Preparation of VSV Protein

Before more detailed biochemical analysis of viral proteins could be readily carried out, it was necessary to obtain relatively large amounts of pure proteins. With the availability of an operating preparative polyacrylamide gel apparatus* at the National Research Council of Canada Laboratories in Ottawa and with the kind assistance of Drs. L. Vinsentin and A. T. Matheson, a sample of VSV protein was subjected to this technique. Two separate runs were performed and the resultant separations of virus protein are shown in Fig. 44. As can be seen the proteins seem to be distributed over a large number of fractions and in some cases there appears to be more than one protein present in any one peak. Reanalysis of some of the fractions on analytical polyacrylamide gels yielded the result shown in Fig. 45. It can be seen that the protein VP_3 appears homogeneous while the material isolated as VP_4 from the preparative column shows an additional protein component which migrated ahead of marker protein VP_4. It is evident that use of preparative columns while potentially of great value will require careful examination before comparisons can be made with analytical gel results. Similar conclusions were also arrived at by Wagner et al. (1969).

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*Shandon

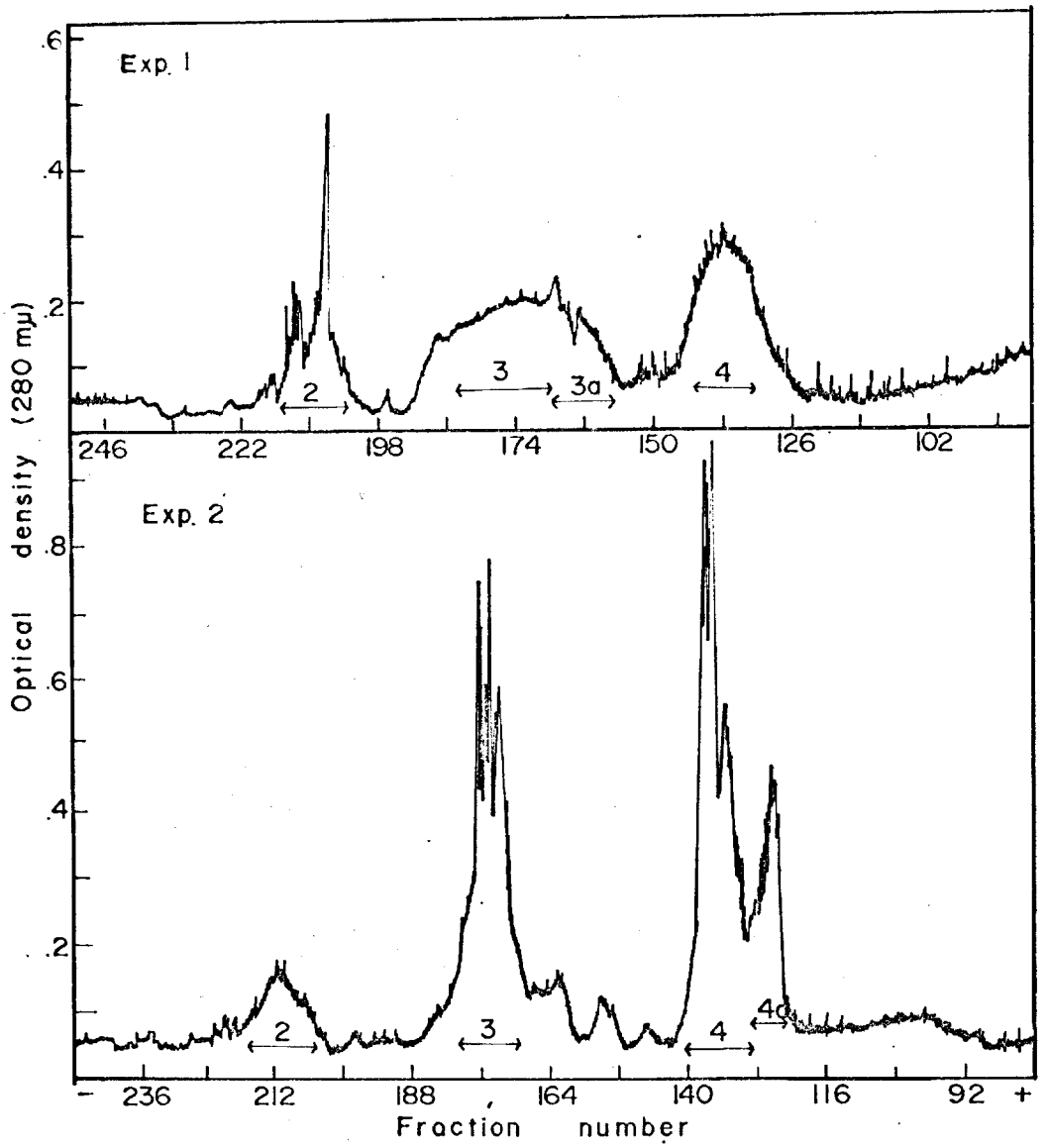

Fig. 44. Preparative polyacrylamide gel electrophoresis of viral proteins.
Samples were placed on a preformed 5% polyacrylamide gel with normal cross-linker and electrophoresed 20 hours. Fractions of 1.5 ml each were collected with effluent ontinuously monitoring for optical density (280 mμ).

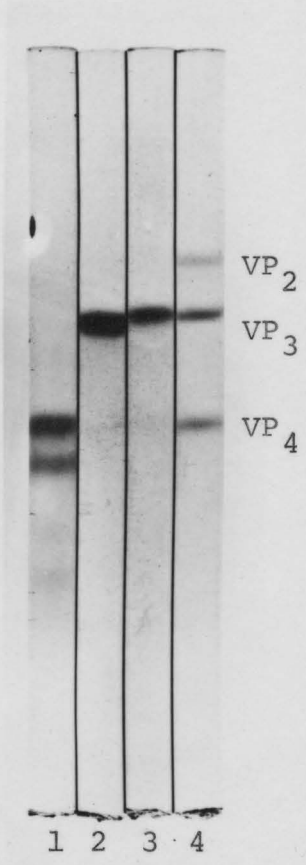

Fig. 45. Analysis of purified viral proteins taken from preparative polyacrylamide gel.
The proteins in each gel are: 1) Experiment 1 - peak 4 in Fig. 44; 2) Experiment 1 - peak 3 in Fig. 44; 3) Experiment 2 - peak 3 in Fig. 44; 4) VSV.
Gels were stained with Coomassive blue.

23. Molecular Weight Estimates from Sedimentation Analysis

In order to confirm the molecular weight estimates from polyacrylamide gel analysis described earlier in this thesis, VSV proteins were also examined by analytical ultracentrifugation. To determine the sedimentation coefficients of the viral proteins, proteins with known molecular weights and sedimentation coefficients were used as standards. Sedimentation runs, development of Schlieren photographs and the relevant measurements were made with the assistance of Mr. A. Peters of the Department of Medicine, McMaster University. Bovine serum albumin pepsinogen, pepsin and trypsin dissolved in 0.01 M phosphate buffer, pH 7.2 containing 0.05 M urea, 0.05% SDS and 0.05% 2-mercaptoethanol yielded uncorrected sedimentation values of 3.65S, 2.43S, 2.14S and 1.35S respectively (Fig. 46). The density of the buffer was carefully determined by repeated weighings of 50 ml of buffer in a volumetric flask, and the relative viscosity by repeated measurements at controlled temperatures in a waterbath using a flow viscosity meter (EXAX). Calculation of the $S_{20.w}$ by the standard formula (Svedberg and Pedersen, 1940) gave values for these proteins which were 25% lower than those previously reported. It is possible that these lower values were due to partial unfolding of the globular proteins by the protein denaturing agents present in the buffer. In any case, the sedimentation coefficients obtained are directly proportional to the

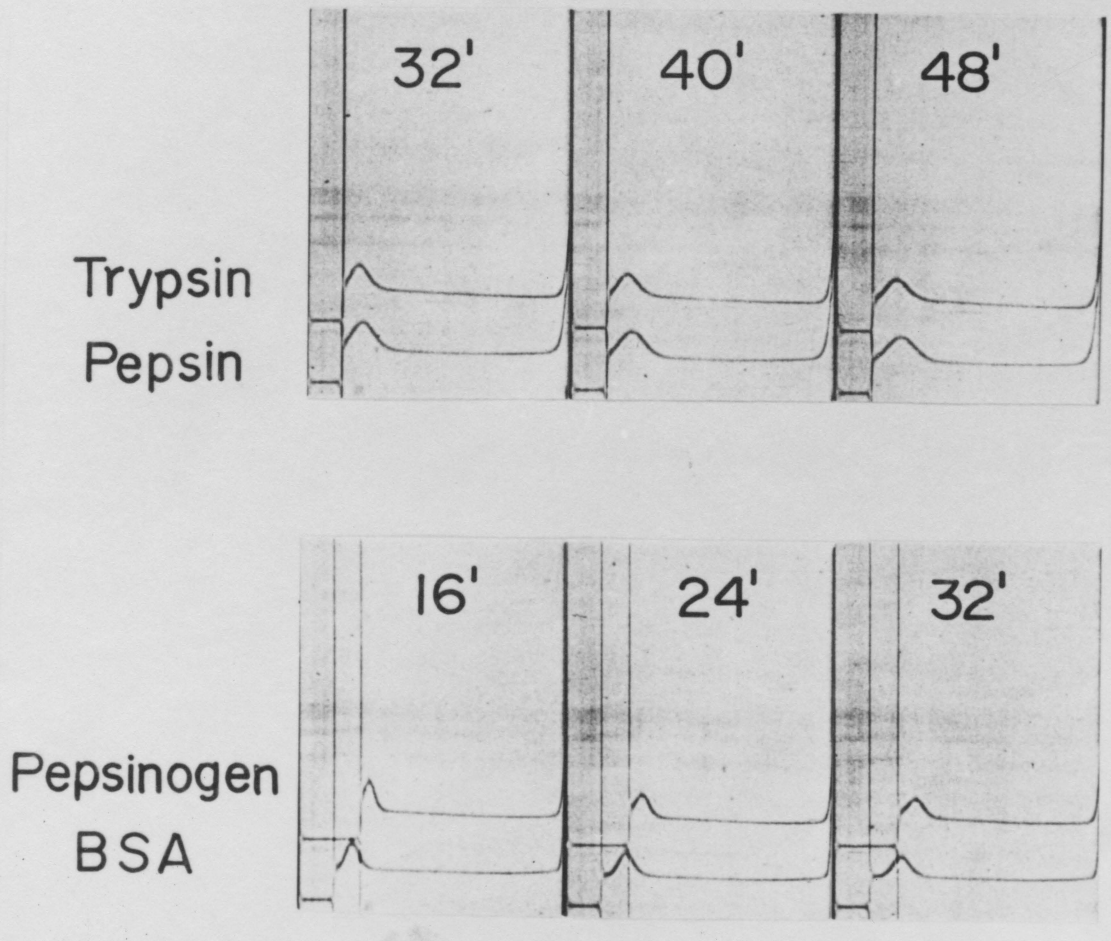

Fig. 46. Schlieren patterns of four reference proteins. Photographs were taken as indicated time (minute) intervals after a speed of 56,000 rpm had been reached in a temperature of 20°C. Sedimentation is towards the right.

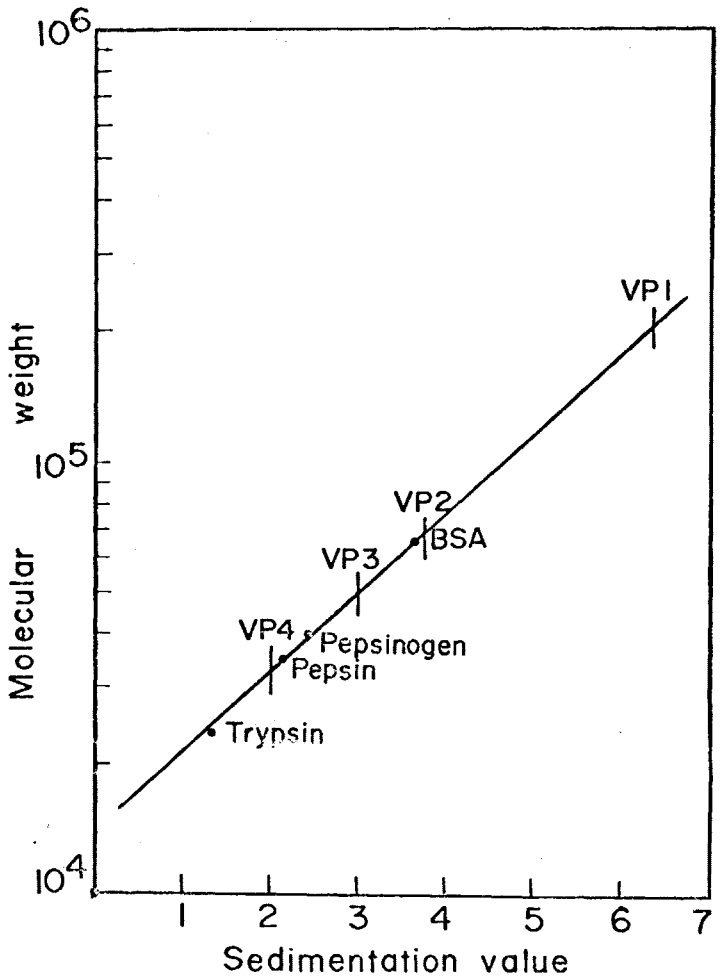

Fig. 47. Determination of molecular weights of the VSV structural proteins by sedimentation velocity. The molecular weights of four reference proteins are taken from Table 1 and the sedimentation coefficients of all proteins are taken from Figures 46 and 48.

logarithm of the molecular weight of the protein (Fig. 47).

The virus proteins were now analyzed under identical conditions. Purified whole virus served as a source of VP_1 since it was assumed that the larger size and hence greater sedimentation coefficient of VP_1 would make it readily separable from the other proteins (Fig. 48). Virus protein VP_2 was obtained from purified virions after treatment with the detergent Nonidet P_{40} as described previously. Virus proteins VP_3 and VP_4 were both obtained from the material obtained by preparative polyacrylamide gel fractionations as described in the previous chapter. While the material constituting VP_3 was seen to be homogeneous on analytical gels, the fraction composing VP_4 may contain a mixture of proteins and the result is therefore subject to some error. Centrifugation of these proteins under conditions identical to those employed for the standards, yielded uncorrected sedimentation coefficients of 6.35S, 3.76S, 3.02S and 2.03S for VP_1, VP_2, VP_3 and VP_4 respectively. It should be pointed out that duplicate analysis of VP_3 taken from different preparative gels gave completely reproducible results.

Using the relationship between sedimentation coefficient and molecular weight defined by the standards as shown in Fig. 48, gives molecular weight values for VP_1 of 200,000, VP_2 of 69,000, VP_3 of 50,000 and VP_4 of 32,000.

While the experiment suffers from the fact that except

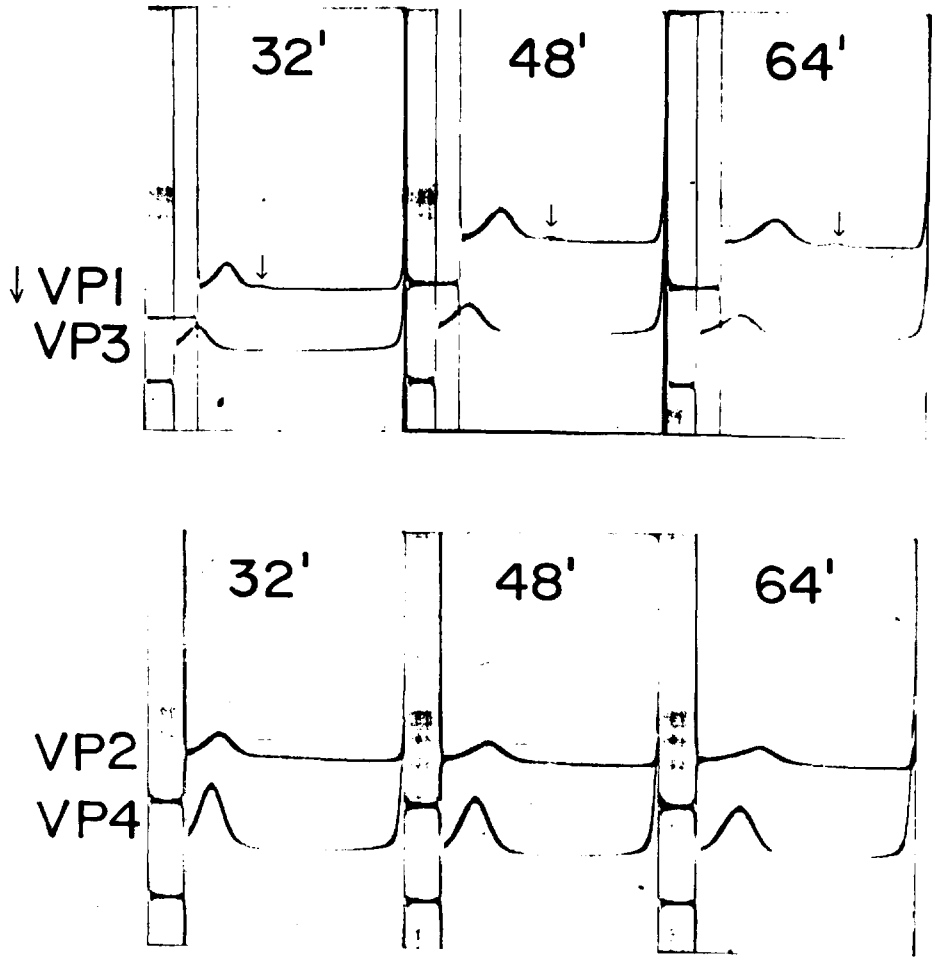

Fig. 48. Schlieren pattern of partially purified viral proteins. Photographs were taken every 16 minutes interval after a speed of 56,000 rpm had been reached in a temperature of 20°C. Sedimentation is towards the right. VP_1 is indicated with arrow mark. The slowly sedimenting peak behind VP_1 represents mixture of VP_2, VP_3 and VP_4.

in one case, only one sedimentation run was made per protein the excellent agreement of the results with those obtained from polyacrylamide gels lend confidence to the result.

24. Amino Acid Composition of VP_3

As was shown, preparative gel procedures yielded large quantities of VP_3 in a homogeneous form. This material was given to Dr. W. W. Chan, Department of Biochemistry, McMaster University for amino acid analysis. Samples for analysis were taken after acid hydrolysis for 24, 48 and 72 hours. Table 4 shows a summary of the results obtained for each hydrolysis time. The frequency of each amino acid in VP_3 polypeptide is represented by the approximately multiples which were calculated to yield a total of amino acids corresponding to the physical molecular weight. The results must remain approximate since no estimate of the tryptophan residues was made.

Table 4

**Purified VP_3 by preparative polyacrylamide gel was precipitated with four volumes of 95% ethanol and dried. A volume of 1 ml of 6 N HCl was added to the sample and hydrolyzed at 110°C for 24, 48 and 72 hours in a evacuated test tube. The amino acid composition of the hydrolyzed polypeptide samples were determined with a Beckman amino acid analyzer.

Table 4

Amino Acid Composition of Virus Protein VP_3

Amino Acids	24 Hours Hydrolysis	48 Hours Hydrolysis	72 Hours Hydrolysis	Average
	(Amino Acid Residues/50,000 molecular weight*)			
Lysine	35.1	31.7	34.6	33.8
Histidine	7.5	8.0	7.2	7.6
Arginine	12.7	13.8	14.8	13.8
Aspartic acid (+ Asparagine)	61.0	56.4	51.2	56.2
Threonine	24.2	22.8	21.9	23.0
Serine	41.8	41.7	41.9	41.8
Glutamic acid (+ Glutamine)	26.8	28.7	28.1	28.1
Proline	22.5	24.0	20.3	22.3
Glycine	38.9	36.9	34.4	36.7
Alanine	22.9	26.4	22.0	23.8
Cystine (half)	0	0	0	0
Valine	16.9	14.6	16.1	15.9
Methionine	1.1	1.1	1.7	1.3
Isoleucine	10.4	11.0	16.8	12.8
Leucine	32.4	35.9	40.1	36.1
Tyrosine	20.2	21.3	20.1	20.5
Phenylalanine	19.8	20.0	17.5	19.1

*The molecular weight of VP_3 has been estimated to be approximately 50,000 from polyacrylamide gel analysis and analytical ultracentrifugation.

DISCUSSION

The studies described in this thesis are concerned primarily with the identification and characterization of virus-specific proteins produced during infection. An understanding of the nature and interrelationships of the structural proteins of the virion particles, of the virus-specific soluble antigens and of intracellular proteins are a first step towards defining the biochemical processes involved in viral interference. To this end this discussion shall consider first the observations regarding virus proteins in structural and intracellular components, then the immunological relationships of viral antigens and finally attempt to relate these observations to what has been shown both in this thesis and by others regarding the process of viral interference.

1. Protein Composition of VS Virions

The infectious B particles and the defective LT and ST particles of Indiana VSV contain four major protein components when examined by polyacrylamide gel electrophoresis. In order of increasing mobility, viral proteins are designated VP_1, VP_2, VP_3 and VP_4. This result was confirmed later by other investigators (Wagner *et al.*, 1970, Petric

and Prevec, 1970; Cartwright et al., 1970; Burge and Huang, 1970).

The complete correspondance of proteins in each of these particles was not unexpected, since B and T particles appear to be antigenically similar (Huang and Wagner, 1966) and, except for their length, are morphologically identical when observed by electron microscopy (Huang et al., 1966; Petric and Prevec, 1970).

The approximate molecular weight of each of the viral proteins can be estimated after SDS-polyacrylamide gel electrophoresis (Shapiro et al., 1967; Weber and Osborn, 1969) and by analytical ultracentrifugation (Svedberg and Pedersen, 1940). Using various combinations of 18 different protein markers under different gel conditions, the molecular weight of virus proteins VP_1, VP_2, VP_3 and VP_4 are estimated as 190,000, 69,000, 50,000 and 29,000 respectively. The excellent agreement between these results and those determined from sedimentation velocity values should be noted.

The protein VP_1, if it is a single continuous polypeptide chain, would account for approximately one-half of the genetic information of the B particle genome. The higher molecular weight values of 275,000 to 300,000 ascribed to this protein by Wagner et al. (1969) and Burge and Huang (1970) are probably due to the error present in extrapolating molecular weight values to regions of low mobility in

polyacrylamide gels. Both Wagner et al. (1970) and Mudd and Summers (1970), on the basis of their molecular weight determinations or from a consideration of the number of virus-specific proteins produced in infection, concluded that there was insufficient viral genetic information available for the synthesis of VP_1 and therefore that VP_1 was an aggregational artifact. The work in this thesis shows quite clearly that a viral genome of 3.6 to 4 x 10^6 could in fact code for the synthesis of all virus-specific proteins produced in infection including VP_1. To date there is no published disaggregation of VP_1. While it is obvious that more direct biochemical work is required to establish the uniqueness of VP_1 it is nevertheless of some interest to examine its occurrence. After dissociation of virions with DOC, VP_1 is not detected in either the ribonucleoproteins core fraction or in the soluble protein fraction. Dissociation of virus with the detergent Nonidet P_{40} however results in the production of a skeleton containing the nucleoprotein core and VP_1 as shown by Cartwright et al. (1970) and the work in this thesis.

The further observations in this thesis that cytoplasmic particles of infected cells contain VP_1 in a DOC sensitive association with VP_3 and NS1 suggest that VP_1 has an intimate relationship to virion ribonucleoprotein. VP_1 may therefore reside inside the viral envelope, possibly

attached to the internal ribonucleoprotein core complex in a manner similar to that of RNA-dependent DNA polymerase of Rous Sarcoma Virus (Coffin and Temin, 1971). Of particular interest in this regard is the finding by Shedlarski and Galet in our laboratory (personal communication) that virus-specific RNA polymerase activity coincides in sucrose gradients with the position of the cytoplasmic particles and that the activity is abolished by DOC treatment. Whether VP_1 is or is not a single polypeptide chain all the evidence suggests that rather than being an artifact of protein aggregation it may well be a functional virus-specific unit.

Virus protein VP_3 appears to be derived principally from the nucleoprotein core of the virus. Nakai and Howatson (1968) described the core as a chain of repeating subunits (90 Å x 30 Å x 30 Å) strung together along the viral RNA strand. Assuming a protein density of 1.3 g/cc, one can calculate that a protein of this size would have a molecular weight of 63,000. The good agreement of this calculated value to the estimate from polyacrylamide gel electrophoresis, suggests that each physical subunit, as defined by Nakai and Howatson, may contain one molecule of VP_3. Wagner *et al.* (1969), Petric and Prevec (1970) and Cartwright *et al.* (1970) have confirmed that the VP_3 is associated with the viral nucleoprotein core.

From the analysis of proteins removed from the virion by DOC treatment, it is concluded that VP_2 and VP_4 are derived

from the viral envelope or coat. Whether these represent the only proteinsin the coat is uncertain in view of the presence of some material in the coat fraction following DOC treatment that bears a mobility similar to that of VP_3 (cf. Fig. 19 c). However, the presence of this material may merely be a reflection of the efficiency of DOC treatment. Bradish and Kirkham (1966) described the morphology of the viral coat as seen by electron microscopy. The coat appears to consist of three distinct layers including a fringe of surface projections. Cartwright et al. (1970) have shown that the surface projections are composed of VP_2 which has subsequently been shown to be glycoprotein (Fig. 24 b). It seems unlikely that such a complex structure is composed of only two distinct proteins. We can consider the following alternatives;

(i) Viral proteins VP_2 and VP_4 may each be composed of a number of proteins having electrophoretically similar mobilities and as such would be undetected.

(ii) Some of the viral coat proteins may be derived from cellular membrane proteins formed prior to infection. In this case, cells, whose proteins had been prelabelled for one full doubling time prior to infection would be expected to transfer some of this label to viral progeny. Although detection of such transfer failed, the question remains unresolved, since it was impossible to calculate the amount of transferred label.

(iii) One of the viral coats may be composed entirely of lipoid material. It is known that the virus contains a large proportion of phospholipid (Prevec and Whitmore, 1963). Simpson and Hauser (1966) showed that the outer viral coat can be disrupted with phospholipase c, with the release of structures which they suggest are composed of cholesterol.

Is NS1 also a structural protein?

The question of whether NS1 is in fact a non-structural protein or is also a constitutent of the virion remains to be determined. The fact that NS1 synthesis occurs early in infection and that the rate of exit of this protein from the cell is slower than any of the other virion proteins would tend to suggest an early intracellular function. In agreement with Wagner et al. (1970), most of the NS1 is in the soluble cytoplasmic pool. We have seen that some NS1 is also associated with an intracellular nucleoprotein structure.

Detectable amounts of protein corresponding to NS1 would be observed by staining polyacrylamide gels to which approximately one mgm of total virus protein from purified virus had been applied. Furthermore some minor radioactive peaks have been observed between VP_3 and VP_4 even though the position and quantity was variable in different preparations of virus. Whether this association of NS1 with the virion is necessary for infectivity or results from contamination of

nucleoprotein during development must await evidence as to the function of NS1 in VSV replication.

2. Properties of 20S and 6S Antigens

Brown and Cartwright (1966a) described an antigen with sedimentation coefficient of 20S which had a rosette-like appearance under the electron microscopy and which they suggest is probably derived from the viral coat. In this case, we might have expected the protein of the 20S particle to be identical to either VP_2 or VP_4. A more intriguing possibility rests on the early observation of Bradish et al. (1956) that infectivity is associated with the 20S antigen. In this case, the 20S antigen might be expected to be a nucleoprotein. The biochemical and the immunological data presented here support the hypothesis, that the protein constituent of the 20S antigen is identical to the protein of virus core. It is possible that the 20S antigen may represent fragments of ribonucleoprotein cores, but no RNA can be detected in 20S antigen, either by ^{3}H-uridine labelling or by treating the 20S material with ribonuclease prior to analysis on sucrose gradients. The structure and functional origin of these core-related antigens still remain to be determined.

The 6S antigen appears to be very closely related to the proteins of the viral coat on the basis of its strong CF activity, immune-precipitation ability and serum blocking

power when tested with coat antibody. On the other hand, the glycoprotein VP_{2a}, which constitutes the 6S antigen, shows a mobility on polyacrylamide gels that is slightly different from that of VP_2, the glycoprotein containing fraction from virus particles. There appears to be twice as much glucosamine per unit protein in VP_{2a} as in VP_2. Thus, although VP_2 and VP_{2a} are undoubtedly closely related antigens, they do not appear to be physically identical. Whether the differences are due to the carbohydrate moieties or to the peptide portions of these glycoprotein containing fractions remains to be determined.

As was shown in Fig. 31 b the position in the gel of the virion marker protein VP_2 does not identically correspond with the position of the intracellular VP_2. This protein in the cell always migrates slightly faster on neutral polyacrylamide gels than the corresponding virion protein but not as fast as the soluble glycoprotein VP_{2a}. If mobility is a function of molecular weight under these conditions, then virion VP_2 is larger than cellular VP_2 which in turn is larger than VP_{2a}. A possible mechanism for the formation of these proteins might be that virion VP_2 is formed from cellular VP_2 by glucosylation while the 6S antigen results from both glucosylation and proteolytic action. The kinetics as presented in the Results neither prove nor disprove a scheme of this sort.

3. Kinetics of Virus Protein Synthesis

Unlike some RNA phages or polio virus which employ the virion genome as a polycistronic messenger for viral protein synthesis, VSV appears to use shorter, perhaps monocistronics, messenger molecules, complementary to the viral genome, to code for virus-specific proteins (Petric and Prevec, 1970; Mudd and Summers, 1970a; Huang et al., 1970). Using very short labelling periods and pulse-chase techniques, Wagner et al. (1970) and Mudd and Summers (1970) have shown that VSV proteins probably do not result from proteolytic cleavage of larger precursor polypeptides. Both of these groups, in agreement with the Results in this thesis, observed that the synthesis of virus proteins was not equimolar, suggesting either a different rate of synthesis or of degradation for each viral protein. In addition, this thesis presents an interesting observation that there is a real difference in the rate of synthesis of virus structural and non-structural proteins during the replication cycle. The synthesis of the non-structural protein, NS1, constitutes some 30% of the total virus protein synthesis in the first two and one half hours of infection but only 10% after four hours of infection. This result suggests that there may be a control, perhaps at the transcription level, of the synthesis of virus proteins. The failure of both Wagner et al. (1970) and Mudd and Summers (1970) to observe this result is undoubtedly due to the fact that virus-specific protein

synthesis is obscured by the large amount of cellular protein synthesis which occurs in the first two hours following infection if cells are not pretreated with Act D.

4. Assembly of Virus Proteins

The pulse-chase experiment demonstrates that all of the virus specific proteins, including perhaps NS1, move into the cell-free supernatant at significantly different rates. The internal protein VP_3, which constitutes about 40% of the virus-specific protein synthesis at any time following infection, appears to accumulate within the cell prior to its release; this is consistent with the observations of Wagner et al. (1970). Petric and Prevec (1970) observed that ribonucleoprotein particles accumulated in cells co-infected with infectious and defective particles. Free ribonucleoprotein particles do not exist in cells infected with infectious virus alone, and VP_3 must accumulate in ribonucleoprotein present in the 140S and 160S native-cytoplasmic structure. The delayed exit of VP_3 from the cell can, at least in part, be explained by the time required for the interaction of many molecules of this protein with RNA to form the ribonucleoprotein core.

In contrast to VP_3, the protein VP_4 while it constitutes only about 15% of the total virus protein being synthesized at any time following infection, constitutes some 50% of the radioactive viral protein released from the cell at the end of

a 20 minute labelling period. This result would suggest that newly synthesized VP_4 can be incorporated rapidly into virions soon after its synthesis. The fact that radioactive VP_4 continues to leave the cell at a rate approximately equivalent to that of VP_2 shows that, while VP_4 may exit rapidly, it does not necessarily do so.

The pulse-chase experimental results for the incorporation of newly synthesized viral proteins VP_4 and VP_2 are in direct contrast to those of Wagner et al. (1970). These researchers observed that radioactive VP_2 was released most quickly to the virion and then stayed constant in amount while the amount of radioactive VP_4 continued to increase. Since the results presented in this thesis were reproducible the reason for this discrepancy is not immediately apparent.

5. Immunological Comparisons of Antigens of Both Indiana and New Jersey Serotype

Wagner et al. (1969) have shown that VSV of the New Jersey serotype contains four virus-specific proteins similar to those of the Indiana serotype. The only observable difference on polyacrylamide gel patterns was the higher mobility of VP_4, from the New Jersey virus when compared to the corresponding protein from the Indiana serotype. As previously shown the two serotypes produce distinct neutralizing antibodies (Cotton, 1927) but share a common complement fixing antigen (Myers and Hanson, 1962). As shown in this work these

results are due to the fact that VP_2 and VP_4 are distinct in the two serotypes while the internal protein VP_3 is antigenically similar in both. This situation is analogous to that existing within the type A influenza (Andrew et al., 1955) and the avian tumour viruses (Kellof and Vogt, 1966).

It was interesting to note that antibody directed against the internal protein VP_3 was capable of showing CF activity with sucrose gradient purified virus. This brings up to the problem of how it is possible for an antibody to react with an antigen located in the interior of a virus particle. Since VSV is susceptible to partial degradation during purification procedures (see electron micrograph in Fig. 5), it is possible that the core antibody is reacting with partially disrupted virions which will fix the complement. Alternatively, the antibody and components of complement may be capable of attacking the core protein via the central axial hollow of the virion.

6. Homotypic and Heterotypic Viral Interference

Observations by Huang et al. (1966) and by Sreevalsan (1970) suggest that the functional T particle RNA is required for viral interference.

It is further demonstrated in this thesis that the interfering activities of both long T and short T particles are identical when tested against homotypic B particles. In contrast, while long T particles are as effective in

heterotypic as in homotypic interference, the short T particles are incapable of heterotypic interference. This result suggests that the additional length of polynucleotide present in the long T particle RNA may be necessary for the expression of heterotypic interference.

Both long T and short T particles can be replicated in the presence of homologous infectious particles as helper suggesting that defective particles can serve as template for their own replication, presumably at the RNA replication level. In contrast, heterotypic infectious particles may not assist the replication of defective particles which would suggest that a RNA replicase of one serotype may not be capable of using the heterotypic T particle RNA as template.

7. Possible Cistron Assignments for VSV Genome

The results as discussed in the previous sections show that VP_2, VP_3, VP_4 and NS1 are distinct virus-specific polypeptides. If, as suggested, the protein VP_1 is also a distinct virus-specified polypeptide then information for its synthesis together with that for the above mentioned proteins must reside in the genome of infectious virion. Using the molecular weights of these proteins, genetic information with a minimum molecular mass of 3.8×10^6 daltons would be required for their synthesis. Since this number corresponds to the molecular weight of a single RNA molecule from B particles (Huang and Wagner, 1966; Nakai and Howatson, 1968; Mudd and

Summers, 1970a), these five proteins may constitute the full complement of virus-specific proteins unless more than one RNA molecule is present per virion.

Since T particle can be generated by stocks of plaque-purified B particles it is reasonable to assume that the RNA of defective T particle is produced from information encoded in the B particle genome. The possibility that the T particle contains a piece of cellular RNA has been negated by RNA-RNA annealing procedures of Schaffer et al. (1968) and Schincariol (Ph.D. Thesis, University of Toronto). These workers showed that the RNA of both B and T particles would form hybrids with a 15S species of RNA produced in cells following infection. The same workers showed that mixtures of B and T particle RNA alone would not form hybrids. These results suggest therefore that the RNA in the T particle is a similar portion of the RNA genome contained in the B particle.

Early in the course of this work, before the molecular weight determinations described in this thesis were obtained and before the protein NS1 was discovered, we proposed as a working model the genome composition presented in Fig. 49 (Prevec and Kang, 1970). This model was based on principally a consideration of the molecular weights of virus-specific proteins and of the RNA's contained by B, LT and ST particles. The location of the genome for NS1 has been left indeterminant in the model. While the more accurate molecular weight determinations of the viral proteins show less correspondence

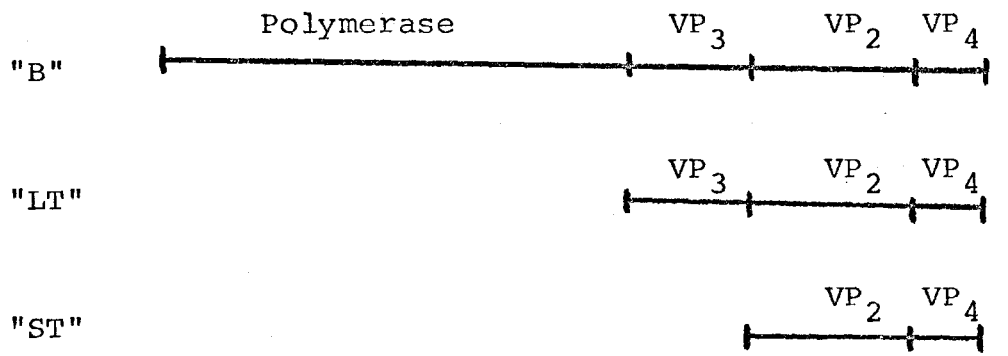

Fig. 49. A proposed model of the cistrons in VSV particle. Apart from the grouping of the cistrons of VP_2 and VP_4 and the grouping of these citrons with that of VP_3 and NS1, the linear arrangement of these cistrons along the genome is arbitrary. The expected cistron sizes are derived from the molecular weights of viral proteins. The molecular weights of the genomes are from Nakai and Howatson (1968) and Petric and Prevec (1970).

between expected cistron size and the determined molecular weights of viral RNA's the model is still worthy of consideration. In the first place, it should be pointed out that the available molecular weight determination for the RNA molecules are still relatively imprecise. Very recently Schincariol (Ph.D. Thesis, University of Toronto) tested RNA-RNA hybrid formation between defective particle RNA and virus-specific polyribosomal RNA molecules presumed to be specific monocistronic messengers for VP_2, VP_3 and VP_4. In agreement with the model the RNA of ST particles hybridized preferentially with the presumed messengers for VP_2 and VP_4 while RNA of LT particles hybridized equally with all three classes of messenger RNA.

Although the model presents the polymerase cistrons as one unit, the replicase and transcriptase functions may be distinct enzymes or different activities of the same enzyme. On the basis of the proposed cistron assignments the defectiveness of T particle is explained by the absence of the polymerase cistron. Replication of T particles in the presence of helper B particles could occur if the viral replicase specified by the B particle genomes is capable of replicating the T particle RNA.

At least two hypotheses for interference have been previously proposed. One suggests (Huang and Wagner, 1966) that a product of the T particle genome may act as an inhibitor of an essential "early" protein coded for by the B particle genome. The second hypothesis suggests that the T particle

genome may compete with B particle RNA as a template for available polymerase molecules (Stampfer et al., 1969). To distinguish between these possibilities on the basis of the model it will be necessary to determine whether T particles, like B particles (Baltimore et al., 1970), carry a virion-associated transcriptase or if transcription from T particle genome is wholly dependent on an enzyme synthesized from the B particle. In particular, it will be important to know whether long T particle RNA is replicated in the presence of New Jersey B particles.

Regardless of which mechanism is operative in interference the proposed cistron assignments afford a possible explanation of the difference in heterotypic interference exhibited by the two T particles. Because the proteins VP_2 and VP_4 are antigenically distinct in the two VSV serotypes it may reasonably be assumed that the heterologous virus is incapable of recognizing either the RNA of the ST particle or the products of the ST genome and thus no interference occurs. On the other hand the LT particle contains the genome for VP_3, a protein which is antigenically, and therefore presumably structurally, similar in both serotypes. It may then be possible that this segment of the genome allows recognition by the heterologous polymerase leading to transcription or replication and subsequent interference.

At this stage of the work in virus interference it would seem that a detailed analysis of the proposed model would

be warranted as it may well pinpoint the essential step in the interference process.

SUMMARY

Infection of mouse L-cells in culture with infectious "B" particles of vesicular stomatitis virus (VSV) results in the production of not only more B particles but also of shorter, defective "T" particles. Depending on the strain of virus used for infection either short T (ST) or long T (LT) particles may be produced. When added early in infection to cells infected with homotypic B particles, both ST and LT particles cause a reduction in the final B particle yield (interference) and B particle assisted replication of the defective particles can occur. When B particles are of heterotypic origin only the LT particles are capable of producing interference but little if any particle replication occurs in this case.

Along with B and T particles, infection of cells results in the production of two smaller antigens, detected by complement fixing activity (CF) to whole virus antibody and characterized by sedimentation coefficients of 20S and 6S.

The products of VSV infection, labelled with radioactive amino acids during growth, were purified by sedimentation velocity on sucrose gradients and the constituent proteins examined by polyacrylamide gel electrophoresis. B, LT and ST particles were each found to

contain identically four major proteins; VP_1, VP_2, VP_3 and VP_4 numbered in order of increasing mobility. The corresponding molecular weight for each protein respectively was determined to be 190,000, 69,000, 50,000 and 29,000. By examining the proteins of the separated nucleoprotein and envelope components of the virions after disruption with the detergent sodium deoxycholat the protein VP_3 is found in the nucleoprotein core while VP_2 and VP_4 are found in the virus envelope. The 20S antigen contains a single protein species which is antigenically and electrophoretical indistinguishable from VP_3. The 6S antigen contains a single protein species which is antigenically related to the proteins of the virus coat and which like VP_2 is a glycoprotein. The protein of the 6S antigen has a slightly higher mobility in polyacrylamide gels than VP_2.

A comparison of the Indiana and New Jersey serotypes of VSV showed that the coat antigens and the 6S antigen are immunologically distinct while the nucleoprotein and 20S antigens of each serotype showed cross reactivity in CF tests.

An examination of the kinetics of synthesis and assembly of newly synthesized virus specific proteins in VSV infected cells showed that the rate of virus protein synthesis is maximal about four hours post-infection, a constant proportion of the newly synthesized protein being released from the cell at all times. The intracellular VP_2 was shown to be distinct by its mobility on polyacrylamide gels from both the virion glycoprotein VP_2 and the 6S antigen glycoprotein

and may be a precursor to these proteins. An additional virus specific protein (NS1) was identified in infected cells. Exposing cells to Act D prior to infection showed that relative rate of synthesis of NS1 was greatest in the first two hours of infection and decreased at later times. The result suggests that the protein NS1 has an early intracellular function in replication.

Pulse-chase experiments showed that the proteins VP_1 and VP_4 entered virions soon after synthesis whereas newly synthesized VP_2 and VP_3 entered virions only after a delay.

A model of the possible genome composition of the infectious B particle and the defective LT and ST particles is proposed.

APPENDIX

List of Publications

1. C. Y. Kang and L. Prevec. 1969. Proteins of vesicular stomatitis virus. I. Polyacrylamide gel analysis of viral antigens. J. Virol. 3: 404-413.

2. C. Y. Kang and L. Prevec. 1970. Proteins of vesicular stomatitis virus. II. Immunological comparisons of viral antigens. J. Virol. 6: 20-27.

3. L. Prevec and C. Y. Kang. 1970. Homotypic and heterotypic interference by defective particles of vesicular stomatitis virus. Nature 228: 25-27.

4. C. Y. Kang and L. Prevec. 1971. Proteins of vesicular stomatitis virus. III. Intracellular synthesis and assembly of virus-specific proteins. Virology (in press).

5. C. Y. Kang, M. Petric and L. Prevec. 1971. An intracellular ribonucleoprotein complex of vesicular stomatitis virus infection. Virology (in press).

BIBLIOGRAPHY

Andrewes, C. H., Bang, F. B., and Burnet, F. M. (1955). A short description of the myxovirus group (influenza and related viruses). Virol. 1: 176-184.

Arstila, P., Halonen, P. E., and Salmi, A. (1969). Hemagglutinin of vesicular stomatitis virus. Arch. Ges. Virusforsch. 27: 198-208.

Atlas, S. M., and Faber, E. (1956). On the molecular weight of cytochrome c from mammalian heart muscle. J. Biol. Chem. 219: 31-37.

Bader, J. P. (1965). The requirement for DNA synthesis in the growth of Rous Sareoma and Rous-Associated viruses. Virol. 26: 253-261.

Baltimore, D. (1970). Viral RNA-dependent DNA polymerase. RNA-dependent DNA polymerase in virions of RNA tumour viruses. Nature 226: 1209-1211.

Baltimore, D., Huang, A. S., and Stampfer, M. (1970). Ribonucleic acid synthesis of vesicular stomatitis virus. II. An RNA polymerase in the virion. Proc. Nat. Acad. Sci. U. S. 66: 572-576.

Barry, R. D., Ives, D. R., and Cruickshank, J. G. (1962). Participation of DNA in the multiplication of influenza virus. Nature 194: 1139-1140.

Beijerinck, M. W. (1898). Ueber ein contagium virum fluidum als ursache der flechenkrankheit der tabaksblatter. Verh. Akad. Wetensch, Amsterdam II, 6: 1-21.

Bellett, A. J. D., and Cooper, P. D. (1959). Some properties of the transmissible interfering component of vesicular stomatitis virus preparations. J. Gen. Microbiol. 21: 498-509.

Bergold, G. H., and Munz, K. (1967). Ultrastructure of Cocal, Indiana and New Jersey serotypes of vesicular stomatitis virus. J. Ultrastrut. Res. 17: 233-244.

Bradish, C. J., Brooksby, J. B., and Dillon, J. R. Jr., (1956). Biophysical studies of the virus system of vesicular stomatitis. J. Gen. Microbiol. 14: 290-314.

Bradish, C. J., and Kirkham, J. B. (1966). The morphology of vesicular stomatitis virus (Indiana c) derived from chick embryos of cultures of $BHK_{21/13}$ cells. J. Gen. Microbiol. 44: 359-371.

Bray, G. A. (1960). A simple, efficient liquid scintillator for counting aqueous solution in a liquid scintillation counter. Anal. Biochem. 1: 279-285.

Brown, F., Cartwright, B., and Almeida, J. D. (1966). The antigens of VSV. I. Separations and immunogenicity of three complement fixing components. J. Immunol. 96: 537-545.

Brown, F., and Cartwright, B. (1966a). The antigens of VSV. II. The presence of two low molecular weight immunogens in virus suspensions. J. Immunol. 97: 612-620.

Brown, F., Cartwright, B., and Smale, C. J. (1967). The antigens of VSV. III. Structure and immunogenecity of antigens derived from the virion by treatment by tween and ether. J. Immunol. 99: 171-177.

Brown, F., Martin, S. J., Cartwright, B., and Crick, J. (1967a). The ribonucleic acids of the infective and interfering components of vesicular stomatitis virus. J. Gen. Virol. 1: 479-486.

Burge, B. W., and Huang, A. S. (1970). Comparison of membrane protein glycopeptides of sindbis virus and vesicular stomatitis virus. J. Virol. 6: 176-182.

Cantell, K., Skurska, Z., Pauker, K., and Henle, W. (1962). Quantitative studies of viral interference in suspended L-cells. II. Factors affecting interference by UV-irradiated Newcastle disease virus against vesicular stomatitis virus. Virol. 17: 312-323.

Cartwright, B., and Pearce, C. A. (1968). Evidence for a host cell component in vesicular stomatitis virus. J. Gen. Virol. 2: 207-214.

Cartwright, B., Smale, C. J., and Brown, F. (1969). Surface structure of vesicular stomatitis virus. J. Gen. Virol. 5: 1-10.

Cartwright, B., Talbot, P., and Brown, F. (1970). The proteins of biologically active sub-units of vesicular stomatitis virus. J. Gen. Virol. 7: 267-272.

Castellino, F. J., and Barker, R. (1968). Examination of the dissociation of multichain proteins in guanidine hydrochloride by membrane osmometry. Biochemistry 7: 2207-2217.

Chamsy, H. M., and Cooper, P. D. (1963). Indirect evidence for the presence of ribonucleic acid in vesicular stomatitis virus. Virol. 20: 14-19.

Chow, T. L., Chow, F. H., and Hanson, R. P. (1954). Morphology of vesicular stomatitis virus. J. Bact. 68: 724-726.

Coffin, J. M., and Temin, H. M. (1971). Comparison of Rous Sarcoma virus specific deoxyribonucleic acid polymerases in virions of Rous Sarcoma virus and in Rous Sarcoma virus-infected chicken cells. J. Virol. 7: 625-634.

Cohen, P. (1971). Department of Biochemistry, University of Washington, Seattle, Washington 98105. Personal communication.

Cole, C. N., Smoler, D., Wimmer, E., and Baltimore, D. (1971). Defective interfering particles of poliovirus. I. Isolation and Physical properties. J. Virol. 7: 478-485.

Cooper, P. D., and Bellett, A. J. D. (1959). A transmissible interfering component of vesicular stomatisis preparations. J. Gen. Microbiol. 21: 485-497.

Cooper, P. D., Summers, D. F., and Maizel, J. V. (1970). Evidence of ambiguity in the post-translational cleavage of poliovirus proteins. Virol. 41: 408-418.

Cotton, W. E. (1926). Vesicular stomatitis. J. Am. Vet. Med. Assoc. 69: 313-320.

Cotton, W. E. (1927). Vesicular stomatitis. Vet. Med. 22: 169-175.

Creeth, J. M. (1958). Sedimentation and diffusion studies on insulin: the maximum molecular weight. Biochem. J. 53: 41-47.

David-West, T. S., and Labzoffsky, N. A. (1968). Electron microscopic studies on the development of vesicular stomatitis virus. Arch. Ges. Virusforch. 23: 105-125.

Ditchfield, J., and Almeida, J. D. (1964). The fine structure of cocal virus. Virol. 24: 232-235.

Dulbecco, R., and Vogt, M. (1954). Plaque formation and isolation of pure lines with poliomyelitis viruses. J. Exptl. Med. 99: 167-182.

Earle, W. R. (1943). Production of malignancy in vitro. J. Natl. Canc. Inst. 4: 165-212.

Edsall, J. T. (1953). The proteins, 1st ed., Neurath and Bailey, Eds., Academic Press, New York. p. 549.

Egnström, L. (1961). Studies on calf-intenstinal alkaline phosphatase. I. Chromatographic purification microheterogeneity and some other properties of the purified enzyme. Biochim. Biophys. Acta. 52: 36-48.

Federer, K. E., Burrows, R., and Brooksby, J. B. (1967). Vesicular stomatitis virus: the relationship between some strains of the Indiana serotype. Res. Vet. Sci. 8: 103-117.

Ferris, D. H., Hanson, R. P., and Dickie, R. (1955). Vesicular stomatitis virus. J. Inf. Dis. 96: 184-188.

Fujinaga, K., Parsons, J. T., Beard, J. W., Beard, D., and Green, M. (1970). Mechanism of carcinogenesis by RNA tumor viruses. I. An RNA-dependent DNA polymerase in murine sarcoma viruses. Proc. Nat. Acad. Sci. U. S. 67: 385-393.

Gazith, J., Himmelfarb, S., and Harrington, W. F. (1970). Studies on the subunit structure of myosin. J. Biol. Chem. 245: 15-22.

Grimes, W. J., and Burge, B. W. (1971). Modification of sindbis virus glycoprotein by host specified glycosyl transferases. J. Virol. 7: 309-313.

Hackett, A. J. (1964). A possible morphologic basis for the autointerference phenomenon in vesicular stomatitis virus. Virol. 24: 51-59.

Hackett, A. J., Zee, Y. C., Schaffer, F. L., and Talens, L. (1968). Electron microscopic study of the morphogenesis of vesicular stomatitis virus. J. Virol. 2: 1154-1162.

Hanson, R. P. (1952). The natural history of vesicular stomatitis. Bacteriol. Rev. 16: 179-204.

Heine, J. W., and Schnaitman, C. A. (1969). Fusion of vesicular stomatitis virus with the cytoplasmic membrane of L-cells. J. Virol. 3: 619-622.

Henle, W. (1963). Interference and interferon in persistent viral infections of cell cultures. J. Immunol. 91: 145-150.

d'Herelle, F. (1917). Sur un microbe invisible antagoniste des bacilles dysenteriques. C. R. Acad. Sci. 165: 373-375.

Howatson, A. F., and Whitmore, G. F. (1962). The development and structure of vesicular stomatitis virus. Virol. 16: 466-478.

Huang, A. S., and Wagner, R. R. (1965). Inhibition of cellular RNA synthesis by non-replicating vesicular stomatitis virus. Proc. Nat. Acad. Sci. U. S. 54: 1579-1584.

Huang, A. S., Greenwalt, J. W., and Wagner, R. R. (1966). Defective T particles in vesicular stomatitis virus. I. Preparations, morphology and some biologic properties. Virol. 30: 161-172.

Huang, A. S., and Wagner, R. R. (1966). Defective T particles of vesicular stomatitis virus. II. Biologic role in homogolous interference. Virol. 30: 173-181.

Huang, A. S., and Baltimore, D. (1970). Defective viral particles and viral disease processes. Nature 226: 325-327.

Huang, A. S., and Stampfer, M. (1970). Vesicular stomatitis virus specific mRNA and polyribosomes. Bacteriol. Proc. 70: 182.

Huang, A. S., Baltimore, D., and Stampfer, M. (1970). Ribonucleic acid synthesis of vesicular stomatitis virus. III. Multiple complementary messenger RNA molecules. Virol. 42: 946-957.

Hutcheon, D. (1898). Aptha or vesicular stomatitis of horse. Vet. J. 47: 54-59.

Jonkers, A. H., Shope, R. E., Atkin, T. H. G., and Spence, L. (1964). Cocal virus, a new agent in Trinidad related to vesicular stomatitis virus, type Indiana. Am. J. Vet. Res. 25: 236-242.

Kawahara, K., and Tanford, C. (1966). The number of polypeptide chains in rabbit muscle aldolase. Biochemistry 5: 1578-1584.

Kellof, G., and Vogt, P. K. (1966). Localization of avian tumour virus group-specific antigen in cell and virus. Virol. 29: 377-384.

Loeffer, F., and Frosch, P. (1898). Berichte der Kommission zer Erforschung der Mual und Klauenseuche bei dem Institut fur Infectionskrankheiten in Berlin. Zbl. Bakter. Abt. I. Orig. 23: 371-391.

Madsen, N. (1971). Department of Biochemistry, University of Alberta, Edmonton 7, Alberta. Personal communication.

Mak, S. (1971). Defective virions in human adenovirus type 12. J. Virol. 7: 426-433.

McClain, M. E., and Hackett, A. J. (1958). A comparative study of the growth of vesicular stomatitis virus in five different tissue culture systems. J. Immunol. 80: 356-361.

McCombs, R. M., Benyesh-Melnick, M., and Brunschwig, J. P. (1966). Biophysical studies of vesicular stomatitis virus. J. Bact. 91: 803-812.

Mohler, J. R. (1918). Mycotic stomatitis of cattle. J. Am. Vet. Med. Assoc. 52: 410.

Morse, D. E., Chan, W., and Horecker, B. L. (1967). The subunit structure and carboxy-terminal sequence of rabbit muscle aldolase. Proc. Nat. Acad. Sci. U. S. 58: 628-634.

Mudd, J. A., and Summers, D. F. (1970). Protein synthesis in vesicular stomatitis virus-infected HeLa cells. Virol. 42: 328-340.

Mudd, J. A., and Summers, D. F. (1970a). Polysomal ribonucleic acid of vesicular stomatitis virus-infected HeLa cells. Virol. 42: 958-968.

Mussgay, M., and Weibel, J. (1963). Electron microscopic studies on the development of vesicular stomatitis virus in KB cells. J. Cell Biol. 16: 119-124.

Myers, W. L., and Hanson, R. P. (1962). Immuno-diffusion studies on the antigenic relationships within and between the serotypes of vesicular stomatitis virus. Am. J. Vet. Res. 23: 896-899.

Nakai, T., and Howatson, A. F. (1968). The fine structure of vesicular stomatitis virus. Virol. 35: 268-281.

Newman, J. F. E., and Brown, F. (1969). Induced ribonucleic acids in cells infected with vesicular stomatitis virus. J. Gen. Virol. 5: 305-313.

Noll, H. (1967). Characterization of macromolecules by constant velocity sedimentation. Nature 215: 360-363.

Parks, W. P., Casazza, A. M., Alcott, J., and Melnick, J. L. (1968). Adeno-associated sattlite virus interference with the replication of its helper adenovirus. J. Exp. Med. 127: 91-108.

Petric, M., and Prevec, L. (1970). Vesicular stomatitis virus - A new interfering particle, intracellular structures, and virus specific RNA. Virol. 41: 615-630.

Pons, M., and Hirst, G. K. (1969). The single and double stranded RNAs and the protein of incomplete influenza virus. Virol. 38: 68-72.

Prevec, L., and Whitmore, G. F. (1963). Purification of vesicular stomatitis virus and analysis of ^{32}P-labelled viral components. Virol. 20: 464-471.

Prevec, L. (1965). RNA synthesis in L-cells partially synchronized with FUdR. Ph.D. Thesis, Department of Medical Biophysics, University of Toronto.

Prevec, L., and Kang, C. Y. (1970). Homotypic and heterotypic interference by defective particles of vesicular stomatitis virus. Nature 228: 25-27.

Reed, W. (1902). Recent researches concerning the etiology, propagation, and prevention of yellow fever, by the United States Army Commission. J. Hyg. II (2): 101-119.

Reich, E., Franklin, R. M., Shatkin, A. J., and Tatum, E. L. (1961). Action of Actinomycin D on cellular nucleic acid synthesis and virus production. Science 134: 556-557.

Rous, P. (1911). Transmission of a malignant new growth by means of a cell-free filtrate. J. Am. Med. Assoc. 56: 198.

Rueckert, R. R. (1965). Studies of the structure of viruses of the Columbia SK group. V. The protein subunits of ME-virus and other membranes of the Columbia SK group. Virol. 26: 345-358.

Rutishauser, U., Cunningham, B. A., Bennett, C., Boningsberg, W. H., and Edelman, G. M. (1968). Amino acid sequence of the Fc region of a human γG immunoglobulin. Proc. Nat. Acad. Sci. U. S. 61: 1414-1421.

Schaffer, F. L., Hackett, A. J., and Soergel, M. E. (1968). Vesicular stomatitis virus RNA: Complementarity between infected cell RNA and RNAs from infectious and autointerfering viral fractions. Biochem. Biophys. Res. Commun. 31: 685-692.

Schincariol, A. L., and Howatson, A. F. (1970). Replication of vesicular stomatitis virus. I. Viral specific RNA and nucleoprotein in infected cells. Virol. 42: 732-743.

Schroeder, W. A., Shelton, J. R., Shelton, J. B., and Olson, B. M. (1964). Some amino acid sequences in bovine-liver catalase. Biochim. Biophys. Acta 89: 47-65.

Schulze, P., and Liebermann, H. (1966). Electronenmikroskopische Untersuchungen zur Morphologie und Entwicklung des virus des stomatitis vesiculosis im Kolbernierenzellkuturen. Archiv. fur Experimentelle Veterinaermedzin. 20: 713-729.

Sellers, R. F. (1955). Growth and titration of the viruses of foot and mouth disease and vesicular stomatitis in kidney monolayer tissue cultures. Nature 176: 547-549.

Sever, J. L. (1962). Application of a microtechnique to viral serological investigations. J. Immunol. 88: 320-329.

Shapiro, A. L., Vinuela, E., and Maizel, J. V. (1967). Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochem. Biophys. Res. Commun. 28: 815-820.

Simpson, R. W., and Hauser, R. E. (1966). Structure components of vesicular stomatitis virus. Virol. 29: 654-667.

Simpson, R. W., Hauser, R. E., and Dales, S. (1969). Viropexis of vesicular stomatitis virus by L-cells. Virol. 37: 285-290.

Slayter, H. S., and Lowey, S. (1967). Substructure of the myosin molecule as visualized by electron microscopy. Proc. Nat. Acad. Sci. U. S. 58: 1611-1618.

Spiegelman, S., Burny, A., Das, M. R., Keydar, J., Schlom, J., Travnicek, M., and Watson, K. (1970). RNA-dependent DNA polymerase activity in oncogenic RNA viruses. Nature 227: 1029-1031.

Spirin, A. S. (1963). Some problems concerning the macromolecular structure of ribonucleic acids. Progress in Nucleic Acid Research. Vol. 1, p. 301. Academic Press, New York.

Sreevalsan, T. (1970). Homologous viral interference induction by RNA from defective particles of vesicular stomatitis virus. Science 169: 991-993.

Stampfer, M., Baltimore, D., and Huang, A. S. (1969). Ribonucleic acid synthesis of vesicular stomatitis virus. I. Species of RNA found in Chinese hamster ovary cells infected with plaque-forming and defective particles. J. Virol. 4: 154-161.

Steinmetz, M. A., and Deal, W. C. Jr. (1966). Metabolic control and structure of glycolytic enzymes. III. Dissociation and subunit structure of rabbit muscle pyruvate kinase. Biochemistry 5: 1399-1405.

Summers, D. F., Maizel, J. W., and Darnell, J. E. (1965). Evidence for virus specific non-capsid proteins in poliovirus infected HeLa cells. Proc. Nat. Acad. Sci. U. S. 54: 505-513.

Sund, H., Weber, K., and Molbert, E. (1967). Dissoziation der Rinderleber-Katalase in ihre Untereinheiten. Eur. J. Biochem. 1: 400-410.

Svedberg, T., and Pedersen, K. O. (1940). The ultracentrifuge. Oxford University Press. pp. 1-478.

Temin, H. M. (1964). The participation of DNA in Rous Sarcoma virus production. Virol. 23: 486-494.

Temin, H. M., and Mizutani, S. (1970). Viral RNA-dependent DNA polymerase. RNA-dependent DNA polymerase in virions of Rous Sarcoma Virus. Nature 226: 1211-1213.

Theiler, S. (1901). Eine contagiose stomatitis de pferds in sud-Afrika. Deuttierarztl. Wochschr. 9: 131-132.

Turco, G. (1959). Changes in cellular RNA during growth of vesicular stomatitis virus in chick cell culture. J. Gen. Microbiol. 21: 510-518.

Uchida, S., and Watanabe, S. (1969). Transformation of mouse 3T3 cells by T antigen-forming defective SV_{40} virions (T particles). Virol. 39: 721-728.

Von Prowazek, S. (1907). Chlamyclozoa. II. Gelbsucht der Seidenraupen. Arch. Protistenkunde 10: 358-364.

Wagner, R. R., Levy, A. H., Snyder, R. M., Ratcliff, G. A., and Hyatt, D. F. (1963). Biologic properties of two plaque varients of vesicular stomatitis virus. J. Immunol. 91: 112-122.

Wagner, R. R., and Huang, A. S. (1966). Inhibition of RNA and interferon synthesis in Krebs-2 cells infected with vesicular stomatitis virus. Virol. 28: 1-10.

Wagner, R. R., Schnaitman, T. A., and Snyder, R. M. (1969). Structural proteins of vesicular stomatitis viruses. J. Virol. 3: 395-403.

Wagner, R. R., Snyder, R. M., and Yamazaki, S. (1970). Proteins of vesicular stomatitis virus: Kinetics and cellular sites of synthesis. J. Virol. 5: 548-558.

Watanabe, Y., Kudo, H., and Graham, A. F. (1967). Selective inhibition of reovirus ribonucleic acid synthesis by cycloheximide. J. Virol. 1: 36-44.

Weber, K., and Osborn, M. (1969). The reliability of molecular weight determinations by dodecyl-sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244: 4406-4412.

Wertz, G. W., and Youngner, J. S. (1970). Interferon production and inhibition of host synthesis in cells infected with vesicular stomatitis virus. J. Virol. 6: 476-484.

Williams, R. C., and Rajagopalan, T. G. (1966). Ultracentrifugal characterization of pepsin and pepsinogen. J. Biol. Chem. 241: 4951-4954.

Yang, Y. J., Stoltz, D. B., and Prevec, L. (1969). Growth of vesicular stomatitis virus on a continuous culture line of antheraea eucalipti moth cells. J. Gen. Virol. 5: 473-483.

Zee, Y. C., Hackett, A. J., and Talents, L. (1970). Vesicular stomatitis virus maturation sites in six different host cells. J. Gen. Virol. 7: 95-102.