

REPLICATION OF VESICULAR STOMATITIS VIRUS IN L CELLS

STUDIES ON THE REPLICATION OF A HEAT RESISTANT STRAIN
OF VESICULAR STOMATITIS VIRUS IN MOUSE L CELLS

By

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A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

May, 1970

MASTER OF SCIENCE (1970)
(Biology)

McMASTER UNIVERSITY
Hamilton, Ontario

TITLE: Studies on the Replication of a Heat Resistant Strain
of Vesicular Stomatitis Virus in Mouse L Cells

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NUMBER OF PAGES: vii, 72

SCOPE AND CONTENTS:

A heat resistant strain (HR-LT) of the Indiana serotype of vesicular stomatitis virus was characterized. Virus specific RNA and protein components of the cytoplasmic extract of cells infected with this strain of virus in the presence and absence of interference, were examined.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. L. Prevec for his guidance, assistance and advice throughout the course of this investigation. Special thanks are due to all my laboratory colleagues, notably C. Y. Kang and H. Galet for their assistance and the interest that they expressed in these experiments.

Thanks are also due to Dr. D. B. Stoltz, Dr. A. Howatson and Mr. T. Bistricki for the valuable assistance with the electron microscopy. I am indebted to Mrs. M. Friedrich for typing this thesis. Finally I wish to extend my gratitude to my family for their encouragement and sacrifices made throughout the course of this investigation.

The financial assistance of the National Cancer Institute is gratefully acknowledged.

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1. General Introduction

The last decade has seen a number of significant advances in the study of the molecular basis of viral function and replication. Among these were the observations that not only was poliovirus RNA capable of acting as a template for protein synthesis in vitro (Darnell 1962), but also that poliovirus RNA in the infected cell was associated with polyribosomes which were actively synthesizing virus capsid protein (Scharff et al. 1963). In 1963 Baltimore and Franklin showed that a specific RNA dependent RNA polymerase was produced in mengovirus infected cells from which it could be isolated. Using virus specific polymerase isolated from RNA phage-infected bacteria, Spiegelman et al. and Weissman et al. in 1968 have shown that the replication of phage RNA appears to be semi-conservative, involving double stranded intermediates which consisted of phage RNA and a complementary strand.

These results have not only explained to a large extent the replicative mechanisms of the structurally simple RNA viruses (RNA phage and mammalian picorna viruses), but have also provided a basis for the study of these mechanisms in the more complex RNA virus systems.

2. Pathologic Effects of Vesicular Stomatitis Virus

(a) Historical Note

The symptoms of a vesicular stomatitis virus (VSV) infection

were first described by Hutcheon in 1884. The naturally susceptible hosts, horses and cattle, show clinical symptoms which resemble those of foot-and-mouth disease but are generally less severe. In 1926, Cotton showed that the disease had a viral origin and succeeded in isolating the virus. It has been postulated by Ferris et al. in 1955 that the transmission of VSV between mammalian hosts is mediated by arthropods.

(b) Serotypes

In his studies in 1927 Cotton demonstrated that strains of VSV isolated in New Jersey and Indiana were immunologically distinguishable. These two strains have since been designated the New Jersey serotype and Indiana serotype of VSV. Cotton's data was confirmed by Myers and Hanson in 1962 who also demonstrated by complement fixation tests that the two viruses possess a cross-reacting antigen. Kang and Prevec in 1970 have shown that the type-specific, virus-neutralizing antibodies react with components of the virus coat, whereas the group-specific antigen is located in the internal nucleo-protein of the virus.

A third serotype of VSV named coccal has been isolated from Trinidad rodents in 1965 by Jonkers. It has, however, not been extensively studied so far.

(c) Growth in Cell Culture

McClain in 1958 found that VSV would replicate in a wide range of vertebrate tissue culture cells. These include monkey kidney and chick embryo cells as well as human HeLa and Earles mouse L cells. VERO cells from Cercopithecus monkey and Chinese hamster ovary cells (CHO) may be

added to the above list through the respective work of Schaffer et al. (1968) and Stampfer et al. (1969). In most of these cell cultures the virus is cytopathogenic and can therefore be titrated. Recent studies by Yang et al. (1969) have shown that the virus will also replicate in moth cells in tissue culture.

(d) Interference

McClain in 1958 reported that continuous passage of VSV in L cells led to the reduction of the infectious virus yield. The study of this phenomenon led Cooper and Bellet in 1959 to postulate an agent in the cell lysate which increases in concentration and reduces the infectious virus yield on repeated, undiluted passage of the cell lysate. They called this agent the transmissible interfering component (T). In 1964 Hackett used the electron microscope to study the particles present in the lysates of infected cells. She noted that the interfering property of the lysate could be correlated with the presence of short virus-like particles in the lysate. She therefore suggested that these truncated particles were responsible for the observed interference with the production of infectious VSV.

3. Physical Characteristics of VSV

(a) Morphology of the Infectious Particle

From shadowed virus preparations examined in an electron microscope, Chow et al. (1954) described VSV as a rod shaped unit 210 m μ long and 60 m μ in diameter. These observations were confirmed by Bradish et al. in 1956 although the dimensions obtained were 175 m μ in length and 69 m μ

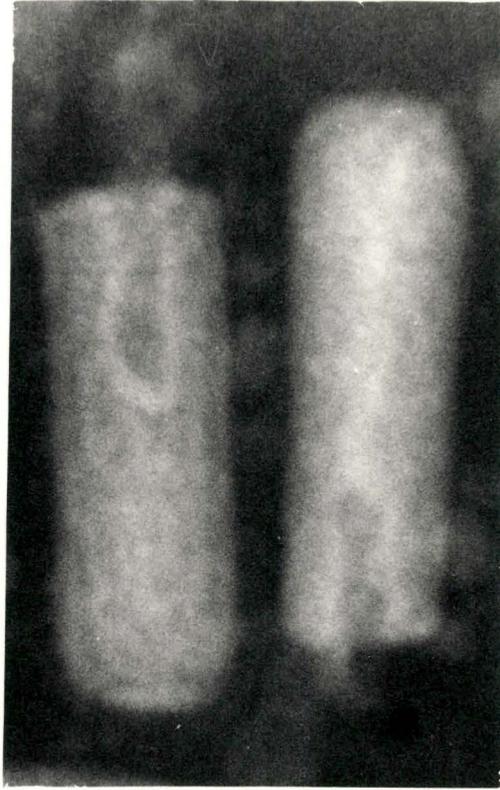


fig. 1

Electron micrograph of VSV.

The preparation was negatively stained with phosphotungstic acid and examined on a Zeiss EM-9 electron microscope.

Mag. X370,000.

in diameter. These authors also determined that the infectious particle had a sedimentation coefficient of 625S. From an electron microscope study of negatively stained virus as well as thin sections of virus-infected cells Howatson and Whitmore in 1962 reported that the cylindrical virus particles were spherical at one end, planar at the other, and covered with filamentous projections 10 μ long. A photomicrograph of the virus is shown in fig. 1. The virus possesses a helical internal component inside which there is a phosphotungstic acid - penetrable central axial hollow about 17 μ in diameter.

The name, B particle, given to the above bullet-shaped infectious particle by Huang and Wagner in 1966, will be used in this thesis.

(b) Isolation and Characterization of the Interfering Particle

In their biophysical studies on VSV Bradish et al. in 1956 observed a nearly spherical non-infectious component about 65 μ in diameter and having a sedimentation coefficient of 330S present in virus-infected-cell lysates. Howatson and Whitmore (1962) believed that similar components present in their virus preparations may have resulted from a breakdown of B particles. On centrifuging a ^{32}P labelled cell lysate in caesium chloride density gradients Prevec and Whitmore (1963) observed, but did not further characterize, a non-infectious component of a lower density than the infectious virus.

As previously mentioned, Hackett in 1964 first observed the correlation between the number of small particles in an infected cell lysate and the interfering activity of that lysate. The first direct evidence for this relationship came from the work of Huang et al. in 1966. After partially purifying the small defective particles by rate

zonal centrifugation on a sucrose gradient, the above workers showed that these particles could be used to interfere with the production of infectious particles. Further characterization of this "transmissible interfering component" or T particle by electron microscopy showed it to resemble the rounded end of the B particle. This T particle which has an almost spherical caplike appearance was 65 μ in length and 65 μ in diameter.

These results were confirmed for the Indiana serotype of VSV by Crick et al. in 1966 and for the New Jersey serotype by Hackett et al. in 1967.

(c) Structure of the Viral Internal Ribonucleoprotein

The idea that VSV contains an internal helix has existed almost since the virus could be seen with an electron microscope. Bradish in 1956 postulated that the internal component of VSV could be "like a coiled spring". Howatson and Whitmore (1962) affirmed that the internal component was helically wound around the central axial hollow, and estimated the pitch of the helix to be 4.5 μ with the entire helix containing 35 turns per B particle. The above results were extended by Hackett in 1964 who showed that the coiled strand making up the helix possessed 24 structural components, each 4.5 μ in width, per turn. Thus in the 34 turns of the helix there would be at least 816 of these subunits.

With this evidence and further electron microscopy Simpson and Hauser in 1966 postulated that the nucleocapsid is made of a single strand 50 Å in width. This strand forms a helical cylinder 500 Å in diameter inside which the same strand forms a smaller helical cylinder about 170 Å

in diameter. Bergold and Munz have compared electron micrographs of VSV with X-ray projections of plexiglass models. They have concluded that the model involving two concentric helices wound over two concentric solid structures was in best agreement with the electron micrographs. Nakai and Howatson in 1968 have found no evidence of the smaller cylindrical helix. They have, moreover, postulated that the long axes of the protein subunits which are radially oriented in the helix while it forms the intact virion, become longitudinally oriented when the helix assumes the smaller diameter as a result of virus degradation.

(d) Chemical and Macromolecular Composition of the B and T Particles

(i) RNA

Evidence that VSV contains RNA was first presented by Chamsy and Cooper (1963) and by Prevec and Whitmore (1963). This RNA was first characterized by Huang in 1966 who showed that RNA extracted from B and T particles had sedimentation coefficients of 43S and 23S respectively. Using the formula developed in 1963 by Spirin $M = 1.550 S_{2,w}^{2.1}$ where M is molecular weight, and S is sedimentation coefficient, the above authors have estimated that the molecular weights of B and T particle RNAs to be 4.0×10^6 and 1.3×10^6 daltons respectively. Nakai and Howatson (1968), from measurements of the length of ribonucleoprotein in the virus particles, have calculated that the molecular weight of B particle RNA is 3.6×10^6 daltons and of the T particle RNA is 1.1×10^6 daltons.

(ii) Proteins

Both B and T particles of Indiana VSV have been shown by Kang and Prevec (1969) and Wagner et al. (1969) to have identical structural

proteins. Analysis of these proteins by polyacrylamide gel electrophoresis revealed four polypeptide species designated VP-1, VP-2, VP-3 and VP-4. It was shown by Wagner et al. (1969b) that VP-3, which has a molecular weight of 59,500 daltons is derived from the viral ribonucleoprotein entity. VP-2 and VP-4, on the other hand, were found to be present in the viral coat. These polypeptides have molecular weights of 81,000 and 34,000 daltons respectively. VP-1, with an estimated molecular weight of 230,000 daltons, has not yet been further characterized. Kang and Prevec (1969) have further shown that VP-2 is, at least in part, a glycoprotein.

The presence of lipids in VSV was suggested by the preliminary studies of Prevec and Whitmore (1963). Cartwright and Pearce (1968) have shown by antigenic studies that a portion of the viral envelope is of cellular origin.

Cells infected with VSV produce not only B and T particles but also low molecular weight antigens which have sedimentation coefficients of 6S and 20S. The 20S antigen was shown by Kang and Prevec (1969) to be antigenically related to virus nucleoprotein and to contain VP-3, whereas the 6S antigen was a glycoprotein which was similar to, but distinct from, VP-2.

4. Effects of Virus Infection on Cell Culture

(a) Effect on Host Macromolecules

Infection with VSV produces a rapid and pronounced decrease in the rate of cellular RNA synthesis. Huang and Wagner (1965) who first

reported this effect showed that neither U.V. irradiation of the virus prior to infection nor the inhibition of protein synthesis with puromycin at the time of infection could abolish this depression of RNA synthesis. The extent of RNA depression was directly proportional to the multiplicity of infection. It was therefore concluded by these authors that the factor responsible for the inhibition of RNA synthesis was a structural element of the infecting virus.

(b) Insensitivity of Virus Replication to Actinomycin D

Actinomycin D (Act. D), an antibiotic which inhibits the synthesis of RNA from DNA templates, has been used extensively in the study of RNA viruses following the work of Reich et al. in 1961. These workers found that while concentrations of Act. D as low as 0.4 μgm per ml. almost completely inhibited RNA synthesis in L cells, much higher concentrations had no effect on the yield of RNA virus. It was shown by Wilson and Bader (1965) that in vitro RNA synthesis mediated by an enzyme found in VSV infected cells was not affected by Act. D concentrations as high as 40 $\mu\text{gm/ml}$. Black and Brown in 1968 showed that while Act. D at 10 $\mu\text{gm/ml}$. inhibited cellular RNA synthesis by 99% it had no effect on the yield of infectious VSV produced by the treated culture.

It should, however, be pointed out that Schaffer in 1968 observed that addition of Act. D along with virus to VERO cell cultures reduced the final virus yield. If actinomycin D was added one hour post infection the yield was normal.

(c) Virus-specific RNA Synthesis in Infected Cells

Schaffer et al. (1968) extracted virus-specific RNA from infected

VERO cells labelled in the presence of Act. D with ^3H -uridine. Analysis of this RNA on sucrose gradients revealed species sedimenting at 43S, 31S, 23S, 15S and 6S. All of these RNA species appeared to be single-stranded judging from their sensitivity to RNase. While it was considered that 43S and 23S RNA species were characteristic of B and T particle RNA respectively, the function of other RNA species was not determined. Hybridization studies performed by these workers showed that the RNAs of B and T particles were not capable of annealing with each other, though both annealed with virus-specific RNA from infected cells. Furthermore, both the 43S and 23S intracellular virus-specific RNA consisted mainly of species which would not hybridize with RNA extracted from virions.

A more recent and extensive study of the VSV specific RNA in infected CHO cells has been done by Stampfer et al. (1969). Infection at a low multiplicity of 1 Plaque Forming Unit (PFU) per cell produced single-stranded RNA species of 43S, 23S and 13S and some partly double-stranded RNA sedimenting at 23S and 35S. At a multiplicity of infection (M.O.I.) of 20 PFU/cell the virus-specific RNA observed early in infection was identical to that for low multiplicity infection. At later times, however, infection at the higher multiplicity showed single-stranded species with sedimentation coefficients of 19S and 6S, partly double-stranded RNA of 19S and 15S and totally double-stranded RNA of sedimentation coefficient 13S. A less extensive study by Newman and Brown (1969) confirmed the above data and provided base ratios for various types of virus-specific RNA present in the cell. These authors also found species of double-stranded RNA sedi-

menting at 11S and 7S present in the virus infected cells. This led them to postulate that VSV RNA could be a complex of four single-stranded 18S units or eight 11S single-stranded molecules, which would give it a sedimentation coefficient of 43S. No further evidence, however, is available to support this hypothesis.

Purpose of this Research Project

It was evident that the replication of VSV was significantly more complex than similar processes involved in mammalian picorna virus or RNA phage. Not only was the problem of T particle production to be explained, but there was also the added problem of a large number of different single-stranded and double-stranded virus specific RNA species synthesized within the infected cell. If an understanding of the defective particle production and viral replication was to be achieved it was first necessary to obtain some information regarding the nature and the associations of these RNA species within the infected cell. From these associations some function for each RNA species could then be deduced.

The progress made in elucidating this problem is described in the present thesis.

MATERIALS AND METHODS

I. MATERIALS

1. Source of Virus

Two strains of the Indiana serotype of VSV were obtained from Dr. A. Howatson of the University of Toronto. Of these, one strain, here designated IND-ST, was identical to the virus used in previously reported work (Nakai and Howatson, 1968). The second strain, designated HR-LT, was selected from IND-ST stocks 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 plaque purified on L cell monolayers.

2. Source of Cells

The cells used in the experiments described in this thesis are a subline of Earles L cells (Earle 1943) designated L-60. These were also obtained from Dr. Howatson.

3. Growth Medium and Other Biological Compounds

Joklik modified minimal essential medium (MEM) was purchased in powder form from the Grand Island Biological Company. 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 . Prior to use the medium was supplemented with sterile fetal calf serum (FCS) also purchased from the Grand Island

Biological Company.

The antibiotic (Act. D) was a gift of Dr. Dorian of Merck Sharp and Dohme (Canada), Ltd.

All radioactive compounds were purchased from the New England Nuclear Corporation. These included ^3H -uridine (29 c/m mole), ^{14}C -uridine 0.85 $\mu\text{c}/\text{m}$ mole, ^3H -amino acids mixture and ^{14}C -amino acids mixture (1.5 mc/gm.).

4. Chemicals

Sodium deoxycholate was purchased from Sigma Biochemicals. Pancreatic RNase and yeast RNA were purchased from General Biochemicals Inc. and Mann Research Laboratories Inc., respectively.

Compounds used in separation and analytical techniques were purchased from Fischer Chemical Co., Toronto, while acrylamide, NN methylene bis acrylamide, NNN'N' tetramethylethylenediamine and β -mercaptoethanol were purchased from Eastman Organic Chemicals, Ltd., New York.

5. Solutions

(a) Solutions used in the work with ribosomes included the following:

i) Phosphate buffered saline (PBS) (Dulbecco 1954)

NaCl	8.0 gm.
KCl	2.0 gm
NaHPO_4	1.15 gm.
KH_2PO_4	2.0 gm.

made up to 1 liter with glass distilled water.

ii) Reticulocyte standard buffer (sodium salt).

RSB⁰ (Prevec 1965)

NaCl	0.58 gm.
MgCl ₂ .6H ₂ O	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.

iii) RSB⁺ - Similar to RSB⁰ but containing a 10 fold concentration of NaCl and MgCl₂.6H₂O.

iv) STE buffer (Prevec 1965)

NaCl	5.8 gm.
EDTA	0.37 gm.
Tris	1.21 gm.

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

(b) Solutions used in polyacrylamide gel electrophoresis:

i) Sodium dodecyl sulphate-mercaptoethanol solution (SDS--MCE) (Burge, 1969)

SDS	1 gm.
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β -mercaptoethanol (100%)	1 ml.
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made up to 100 ml. with glass distilled water.

ii) Virus disrupting buffer (Summers 1965)

SDS	1.0 gm.
Acetic acid (glacial)	10.0 ml.
Urea	30.0 gm.
KH ₂ PO ₄	0.039 gm.
Na ₂ HPO ₄	0.134 gm.

made up to 100 ml. with glass distilled water.

iii) Polyacrylamide gel running buffer (Summers 1965)

SDS	1.0 gm.
KH ₂ PO ₄	0.39 gm.
Na ₂ HPO ₄	1.34 gm.

made up to 1 liter with glass distilled water.

iv) Dialysis buffer (Summers 1965)

SDS	0.9 gm.
Urea	30.0 gm.
β -mercaptoethanol (100%)	1 ml.
Polyacrylamide gel running buffer	100 ml.

made up to 1 liter with double distilled water.

v) Acrylamide Gel Solutions

Solution A

Acrylamide	10.0 gm.
NN methylene bisacrylamide	0.2 gm.

made up to 100 ml. with running buffer.

Solution B

NNN'N' methyltetraethylenediamine	0.1 ml.
Running buffer	100 ml.

Solution C

Ammonium persulphate	0.56 gm.
Running buffer	100 ml.

(b) Solutions used in liquid scintillation counting:

i) Bray's solution (Bray 1960)

2,5 diphenyloxazole (PPO)	12.0 gm.
1,4-bis(2(5 phenyloxaxoly)benzene (POPOP)	0.6 gm.
Methanol	300.0 ml.
Naphthalene	180.0 gm.
Ethylene glycol	60 ml.

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

ii) Toluene based fluid

PPO	4.0 gm.
POPOP	0.3 gm.
Toluene	1.0 liter

II. METHODS

1. Cell Culture

Minimal essential medium (MEM) supplemented with 5% fetal calf serum (FCS) was used to grow a subline of Earles L cells in a suspension culture. This cell suspension was kept in a tightly stoppered erlenmeyer flask containing a sterile teflon coated bar magnet and incubated in a 37°C water bath equipped with magnetic stirrers. The cell concentration measured on a Levy ultraplane hemocytometer was kept between 4 to 7 X 10⁵ cells per ml. by daily dilution of the culture with the above medium.

2. Preparation of Virus Stocks

Cells to be infected were collected from a growing culture by centrifugation at 600 X g at room temperature for 10 minutes in a PR-2 IEC centrifuge. The cell pellet was resuspended to 10⁷ cells/ml. in MEM containing a desired amount of infectious virus. The cells, kept in suspension by a small magnetic stirring bar were incubated at 37°C in the water bath. After an adsorption period of 45 minutes the culture was diluted to 10⁶ cells/ml. with prewarmed MEM supplemented with 2% fetal calf serum and incubated further for 15 to 18 hours.

The lysate was cleared of cells and large cellular debris by centrifugation at 1000 X g for 10 minutes. The supernatant containing the virus was distributed in sterile 12 ml. translucent plastic dilution tubes (Falcon Plastics). A portion of the stock was assayed for infectivity and the remainder stored at -45°C in a Revco freezer.

3. Virus Assay

The infectious virus titer was determined in terms of Plaque

Forming Units per milliliter (PFU/ml.). Monolayers of L cells were prepared by pipetting aliquots of 4×10^6 cells from a suspension culture into sterile Falcon 60 X 15 mm. plastic petri dishes. After 3 hours of incubation at 37°C , 100% humidity and 5% CO_2 in a Hotpack incubator a full monolayer of L cells covered the bottom of each petri dish.

The virus stock was serially diluted in PBS and 0.1 ml. of each dilution was placed on an individual cell monolayer from which the growth medium had been removed. The virus was allowed 45 minutes of incubation at 37°C to adsorb and the monolayer was overlaid with 5 ml. of MEM made up to 5% in FCS, and 0.9% in agar (Difco, Noble). The agar was allowed to solidify at room temperature after which the monolayers were incubated at 37°C in 5% CO_2 for 18 hours. At this time the plaques could usually be seen quite plainly, but to facilitate their counting, the cell sheet was fixed with carnoy fixative (3 vol. of absolute ethanol to 1 vol. of glacial acetic acid), the agar washed away and the cell sheet stained with methylene blue.

4. Labelling of Infected Cells with Radioisotopes

(a) ^3H -Uridine Labelling

About 10^8 cells in exponential growth phase were infected with VSV at an M.O.I. of 50. After a 30 minute absorption period the suspension culture was diluted to 10^6 cells/ml. with MEM containing 2% FCS. The time of this dilution designated the beginning of infection. Act. D (2 $\mu\text{gm}/\text{ml}.$) was added at 30 minutes and ^3H -uridine (2 $\mu\text{c}/\text{ml}.$) at 180 minutes post infection.

(b) ^{14}C -Amino Acid Labelling

Cells infected and treated with Act. D as outlined above were collected by centrifugation at $3\frac{1}{2}$ hours post infection and resuspended in medium containing only $1/20$ the usual concentration of amino acids present in MEM. After 30 minutes of incubation, ^{14}C -amino acids at a concentration of $0.2 \mu\text{c/ml}$. were added to the culture.

In some cases the cell culture was pretreated with $0.5 \mu\text{gm/ml}$. Act. D for 8 hours prior to infection. These cells were then infected and labelled as outlined above, but further Act. D was not added after infection.

5. Extraction of Cytoplasm from Infected Cells

At 5 hours post infection the cells were removed from the medium by centrifugation at 1000 X g for 5 minutes at 4°C in a PR-2 centrifuge and chilled on ice. After the medium was removed, the cells were washed three times by successive resuspension and centrifugation in ice-cold PBS. Following the third washing the cell pellet was resuspended in 1.0 ml . of ice cold RSB^0 for 10 minutes to allow the cells to swell. The cells were then disrupted by 10 strokes of a tight fitting Dounce homogenizer and the homogenate centrifuged at 7000 X g in a Sorvall RC-2B refrigerated centrifuge. This procedure yielded a pellet and a cloudy supernatant.

6. Characterization of the Cytoplasmic Extract on Sucrose Gradients

Linear gradients of 15% to 30% sucrose dissolved in RSB^+ were prepared in 30 or 38 ml . volumes in Beckman nitrocellulose tubes using a

Buchler gradient maker. The gradient tubes were then placed in Beckman SW 25.1 or SW 27 rotor buckets and chilled to 4°C.

The cloudy supernatant obtained from cells disrupted as described above was layered on the top of the sucrose gradients. These were centrifuged at 81,000 X g and 5°C for periods of 2 to 6 hours in an L2-65B ultracentrifuge. The gradients were collected in successive 1 ml. fractions with continuous monitoring of the optical density (254 m μ) of the effluent using an Isco* U.V. analyzer and fraction collector. This apparatus collects a gradient from the lighter portion to the heavier one.

For radioactive analysis a portion of each fraction was made up to 5% (W/V) in ice cold trichloroacetic acid (TCA) and the resultant precipitate collected on nitrocellulose filters (0.45 μ , Sartorius). The precipitate was washed with 5 ml. of ice cold 5% TCA using suction filtration and the filters dried at 120°C. The filters were then immersed in 5 ml. of toluene-based scintillation fluid and radioactivity measured on a Beckman LS-250 liquid scintillation counter.

7. Extraction of RNA from Cytoplasmic Fractions

To further study the RNA contained within a particular cytoplasmic fraction, the fractions of interest were pooled and SDS was added to a final concentration of 0.5%. Two volumes of 95% ethanol were added and the mixture stored at -20°C for 12 hours. The white flocculent precipitate which formed was collected by centrifugation at 12,000 X g for 15 minutes and redissolved in 0.1 ml. of STE containing 0.5% SDS. The solution was layered on a 5 ml. linear gradient of 5% to 20% sucrose dissolved in STE

*Instrumentation Specialties Company, Inc., Lincoln, Nebraska, U.S.A.

containing 0.5% SDS which was preformed in a 2 by ½ inch nitrocellulose tube. At times ^{14}C -uridine labelled ribosomal RNA (5000 cpm) was also added on the gradient to serve as an internal standard for sedimentation. The gradients were centrifuged in an SW50 rotor (Beckman) for 2 hours at 185,000 X g and 15°C. They were then fractionated by dripping 0.16 ml. fractions through a hole punched in the bottom of the nitrocellulose tube. If an internal standard of ^{14}C had been used the fractions were dripped directly onto Sartorius glass fiber filter pads. In other cases the fractions were collected into tubes, and each fraction monitored for optical density (260 m μ) on a Beckman DU spectrophotometer. The material in each tube was then precipitated with ice cold TCA and the precipitate collected on Sartorius nitrocellulose filters. The filters were dried and radioactivity determined as described above.

8. Purification of ^3H -Uridine Labelled Virus and Analysis of Viral RNA

An infected culture was treated with Act. D (2 $\mu\text{gm/ml.}$) at 30 minutes post infection. ^3H -uridine ($\mu\text{c/ml.}$) was added 150 minutes later and the culture harvested after 12 to 20 hours. The cells and debris were removed by centrifugation and the virus was collected from the cell free stock by sedimentation at 41,000 X g for 2 hours in a Sorvall refrigerated centrifuge. The viral pellet was resuspended in 1 ml. of PBS containing 0.5% bovine serum albumin (PBSA), layered on a 30 ml. preformed cold 5% to 30% sucrose gradient in PBSA, and centrifuged in an SW 25.1 rotor at 41,000 X g for 1 hour at 4°C. The gradients were fractionated and the U.V. absorbance of the effluent monitored on an Isco U.V. analyzer and fraction collector. A portion of each fraction was then further analyzed

for radioactivity.

The fractions containing the virus were pooled and the RNA was extracted from them and analyzed on a sucrose gradient as outlined in section 7 above.

9. Polyacrylamide Gel Electrophoresis

(a) Source of Virus Specific Protein

Virus specific protein was obtained from virions, virus infected cell cytoplasmic extract, and virus infected cells. In all cases the radioactive proteins were produced as described in section 4b above. To obtain virion proteins the culture was harvested at 12 to 20 hours post infection and the virus partially purified as described in section 3 above. The virus pellet obtained by centrifugation of the cell free stock, was treated with 0.2 ml. of virus disrupting buffer at room temperature for 1 hour.

Sucrose gradient fractions of the cytoplasmic extract from infected cells, obtained as outlined above, were pooled and dialyzed against 500 volumes of RSB⁺ at 4°C for 8 hours to remove the sucrose, and finally concentrated to near dryness by flash evaporation. The concentrate was treated with 0.2 ml. of virus disrupting buffer for 1 hour at room temperature.

To extract the proteins from whole infected cells, a washed cell pellet from a culture pretreated for 8 to 12 hours with Act. D, infected and labelled with ¹⁴C-amino acids, was treated with 1 ml. of SDS-MCE solution. The pellet was disrupted, and the viscosity of the cell lysate decreased by sonication for 2 minutes at setting 30 on a Bronwill

Biosonic III sonicator. The preparation was kept at room temperature for 1 hour.

(b) Electrophoresis

The above preparations of virus specific protein were dialysed against 1000 volumes of Dialysis buffer for 12 hours. A volume of 0.1 to 0.4 ml., containing at least 40,000 cpm of the dialysed protein solution was brought to 20% in sucrose and layered on a preformed polyacrylamide gel. In some cases 0.1 ml. of the ^{14}C labelled virion protein, also containing 40,000 cpm were also layered on the gel to serve as an internal standard.

To make the gels, Acrylamide gel solutions A, B, C were mixed in a ratio of 4:3:1 respectively and urea was added to a final concentration of 0.5 M. The mixture was then quickly poured to a height of 16 cm. into a tube (0.9 cm. by 25 cm.), closed at one end by a dialysis membrane. The gel was overlaid with 0.5 ml. of distilled water and allowed to stand for 30 minutes to harden.

The protein-sucrose solution was added to the top of the gel and the tubes were carefully filled with running buffer. The tubes were mounted in a Buchler vertical electrophoresis apparatus and the anode of the Buchler power supply connected to the bottom buffer chamber. A potential of 1 volt/cm. was applied to the gel for 30 minutes followed by 3 volts/cm. for a further 15 hours.

The gel was sliced into sections each 0.15 cm. in length. Each slice in a scintillation vial was dissolved in 0.2 ml. of hydrogen peroxide at 50°C in a water saturated atmosphere. Ten ml. of Brays solution

was then added and the radioactivity determined on an LS 250 liquid scintillation counter.

10. Electron Microscopy

A suspension of the material 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.

If the sample contained virions it was washed 3 times by adding a drop of distilled water to the grid and removing it by blotting at the edge. The grid was then covered with 2% phosphotungstic acid (pH 6.8) and after 1 minute the stain was blotted off with filter paper.

If the material from the cytoplasmic fractions was being examined, the grid was washed 10 times with distilled water after application of the sample. Each drop of water was removed by blotting the grid with filter paper from one side only. The material on the grid was negatively stained by adding one drop of 2% uranyl acetate to the grid, waiting for 1 minute, then blotting off the excess stain.

The grids were observed and photographed on Phillips 300 or a Zeiss EM-9 electron microscope.

I. A NEW T PARTICLE

As the heat resistant strain of VSV used in the work outlined in this thesis had not been extensively characterized, an examination of the virus morphology and of the virion-associated RNA of this strain was essential prior to investigating its replication in the infected cell.

1. Morphology of the Heat Resistant Strain of VSV

(a) Sedimentation Properties. The particles present in the lysates of cells infected with the heat resistant strain of VSV were compared by sedimentation on sucrose gradients with those produced by the wild type Indiana serotype of VSV. Cells were infected with both of these strains and Act. D was added to the cell cultures. At 2 hours post infection ^3H -uridine ($2 \mu\text{c}/\text{ml.}$) was added to the cell culture infected with the wild type VSV while ^{14}C -uridine ($0.2 \mu\text{c}/\text{ml.}$) was added to the cells infected with the heat resistant virus. Both cultures were harvested at 14 hours post infection.

The virus particles in the cell free supernatant were collected by centrifugation and each pellet resuspended in 1.5 ml. of PBS containing 0.5% bovine serum albumin (PBSA). One ml. samples of each virus preparation and 1 ml. of a 1:1 mixture of each preparation were separately layered on 5-30% sucrose gradients prepared in PBSA. After centrifugation at 49,000 X g at 5°C for 1 hour the gradients were fractionated, monitored for optical density at 254 m μ and analysed for radioactivity.

The sedimentation pattern of the heat resistant virus particles is seen in fig. 2(A). It has been shown by Kang and Prevec (1969) that the faster sedimenting band (fraction 18) is infectious whereas the other band (fraction 13) is not. The latter particles, however, sediment faster than the wild type T particles in fig. 2(B).

The level of absorbance throughout the sucrose gradient on which the wild type VSV concentrate had been sedimented is shown in fig. 2(B). As this result is very similar to that described by Huang et al. in 1966, the major peaks at fractions 11 and 18 represent the respective sedimentation bands of T and B particles. The shoulder occurring at fraction 13 would, however, have to be due to some intermediate sized particle sedimenting faster than the T particle.

Fig. 2(C) illustrates the sedimentation pattern of particles of the two strains (1:1 ratio) on a sucrose gradient. These results show that the defective T particle of the heat resistant strain has a greater sedimentation coefficient than that of the wild type T particle. While the B particles of both strains sediment together, as evidenced by the superposition of the ^3H and ^{14}C radioactivity in fraction 18, the T particles migrate as distinct peaks. The peak representing the heat resistant strain T particle sediments almost halfway between the T and the B particle of the wild type strain.

From their sedimentation properties the heat resistant strain T particles were considered to be larger than the wild type T particles. Assuming that in the linear sucrose gradient used, the wild type T particles sediment at 330S (Huang et al. 1966) and the B particles at 625S (Bradish

fig. 2 Distribution of virus particles produced by the heat resistant and the wild type Indiana strains of VSV.

Two cell cultures were separately infected with the heat resistant and wild type strains of VSV. Both cultures were treated with Act. D (2 μ gm/ml.) at 30 minutes post infection. One and a half hours later ^{14}C -uridine (0.2 μ c/ml.) was added to the heat resistant strain infected culture and ^3H -uridine (2 μ c/ml.) to the wild type culture. The cultures were harvested after 14 hours, clarified of cells and large debris by low speed centrifugation. The cell-free supernatant was centrifuged at 40,000 X g for 2 hours to pellet the virus. Each virus pellet was resuspended in 1.5 ml. of PBSA of which a 1 ml. volume was layered on a 5 to 30% sucrose gradient in PBS. The remaining virus concentrates were mixed and also layered on a gradient. The gradients were centrifuged for 1 hour at 48,000 X g and collected and the optical density (254 $m\mu$) monitored. The radioactivity in the mixed sample was determined by collecting the TCA precipitated fractions of the gradient on nitrocellulose filters which were dried and monitored in a liquid scintillation counter. ^3H counts (—●—●—●—), ^{14}C counts (- o - o) and optical density (-----) are plotted. The bottom of the centrifuge tube is to the right. (A) heat resistant strain virus, (B) Indiana wild type virus, (C) heat resistant strain and Indiana wild type mixed.

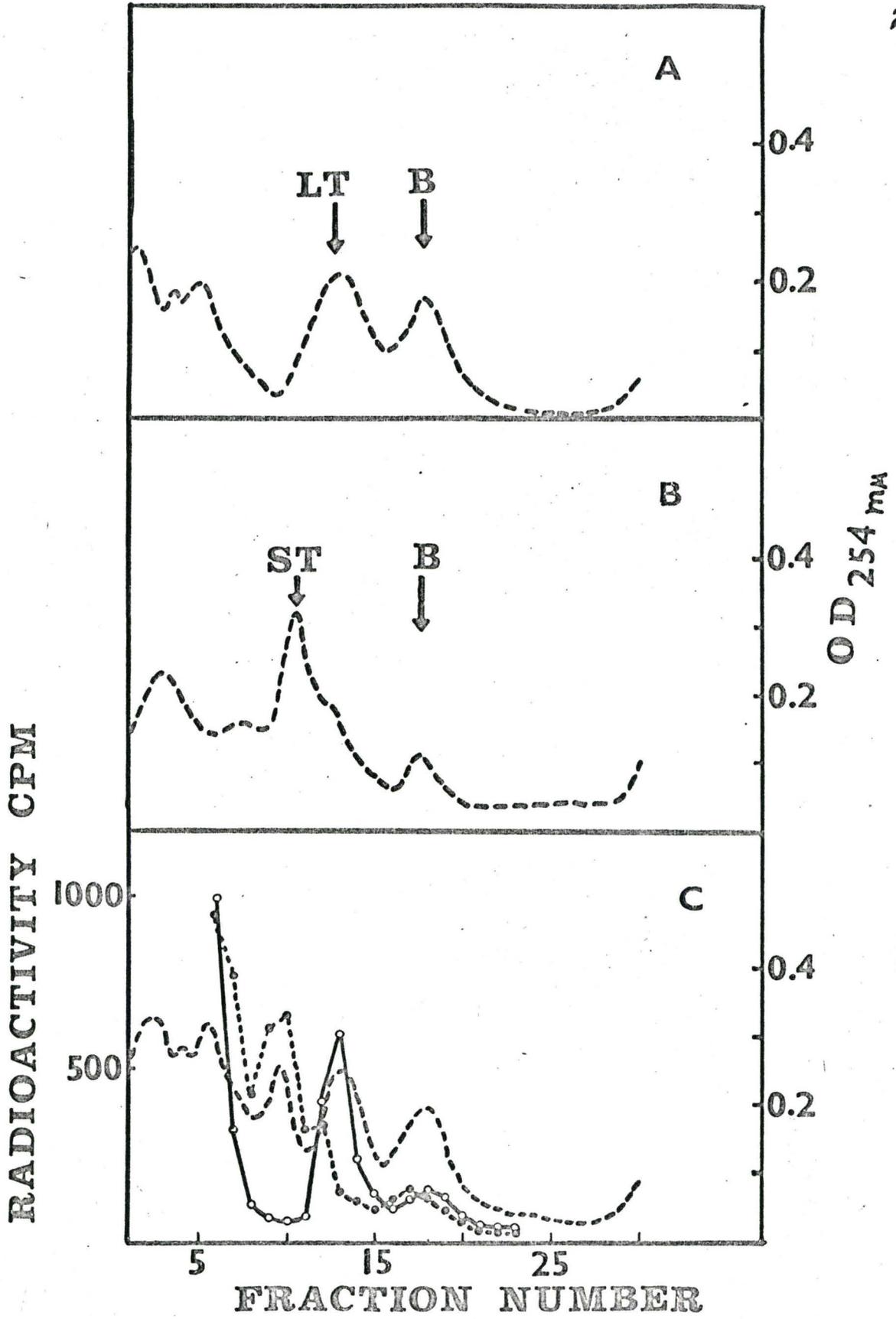
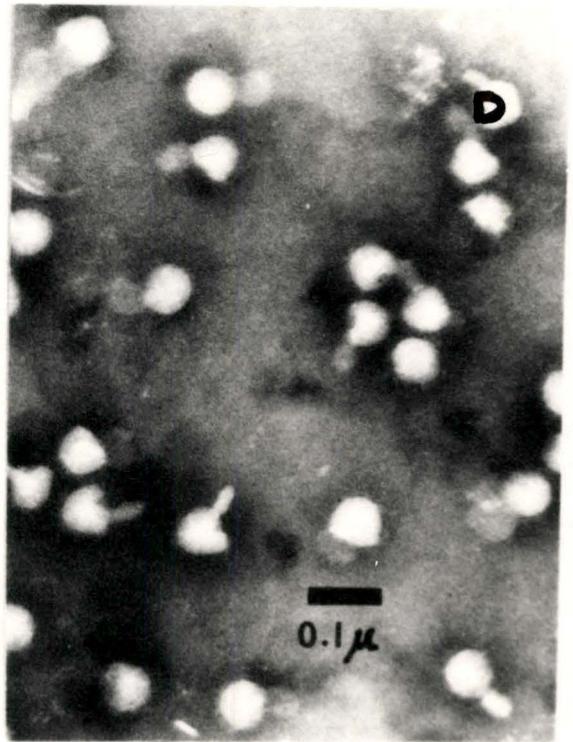
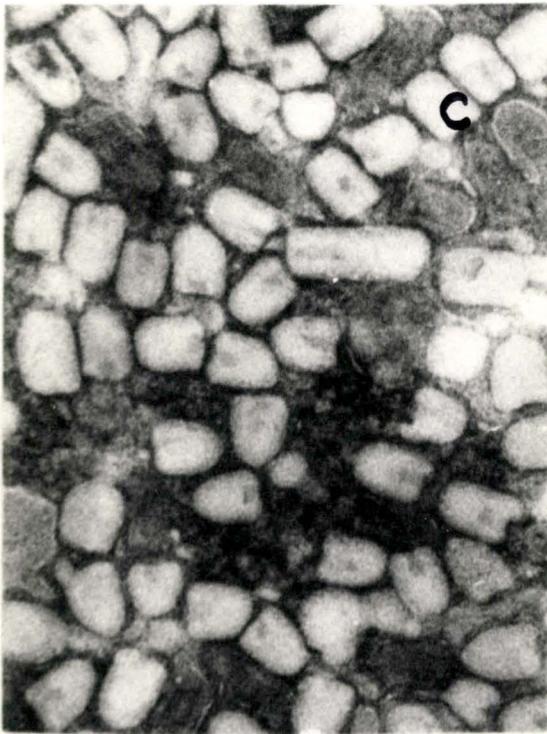
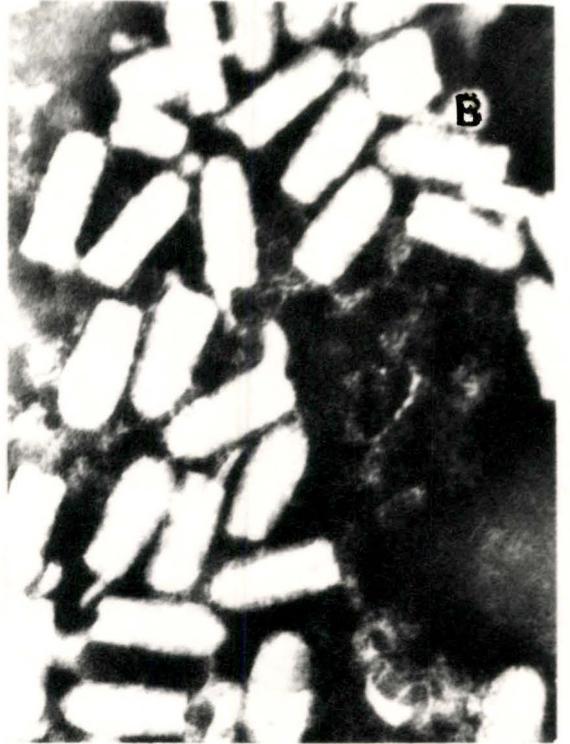
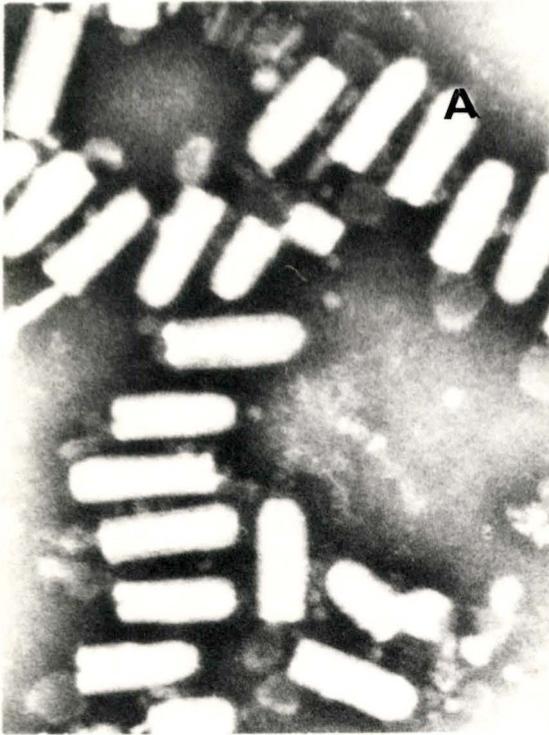


fig. 3 Electron micrographs of B and T particles.

Infectious B particles of the HR-LT strain (a) and of the IND. ST strain (b), defective "long T" particles (c) and short T particles (d) were partially purified and negatively stained with 2% phosphotungstic acid.

Mag. approximately X85,000.



et al. 1955), then the heat resistant strain T particles have an interpolated sedimentation coefficient of 450 to 475S.

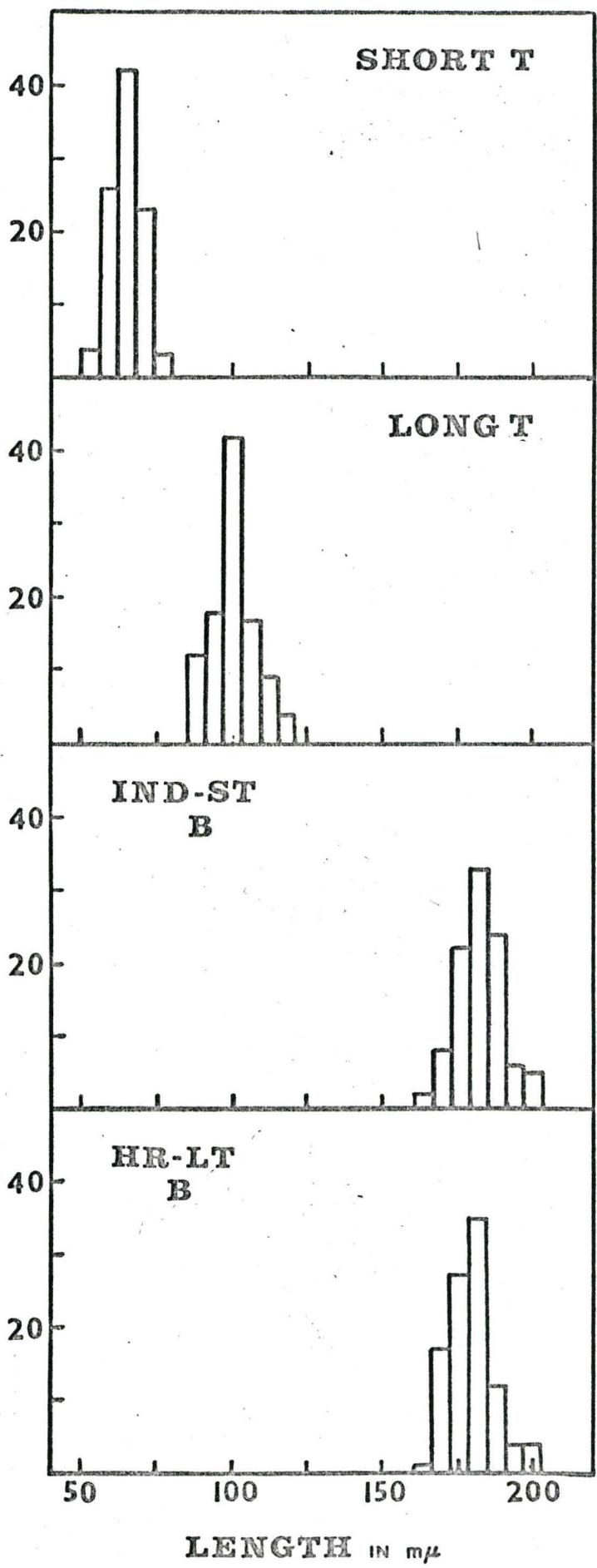
The T particle produced by the heat resistant strain has been shown to produce homologous interference (Prevec and Kang 1970). Hence it is analagous to the transmissible component described by Cooper and Bellet (1959). It has therefore been called long T particle (LT). The heat resistant LT producing strain was termed the HR-LT strain. To clarify the nomenclature the major wild type T particle was designated short T (ST) and the strain producing it IND-ST.

(b) Electron Microscopic Study. Electron micrographs of the material present in each optical density peak discussed above are shown in fig. 3. The B particles of the heat resistant (fig. 3a) and wild type (fig. 3b) strains were found to be of the same size reflecting the fact that these two particles have identical sedimentation coefficients. Because of its greater sedimentation coefficient the heat resistant strain T particle was expected to be longer than the wild type T particle. Electron micrographs (fig. 3c and fig. 3d respectively) show this to be indeed the case.

Accurate length determinations were performed on the LT particles. To do this 100 of these particles were measured from electron micrographs like those in fig. 3c. For purposes of comparison this procedure was repeated for the remaining three types of particles. The distribution in length for each type of particle is shown in fig. 4. The B and ST particles are 180 and 65 μ m long respectively. These values correspond closely to those of Howatson and Whitmore (1962) and Huang et al. (1966). The B

fig. 4 Histograms of the length distribution of the defective T particles and infectious B particles. Electron micrographs similar to those in fig. 3 were used to obtain particle lengths.

NUMBER OF PARTICLES



LENGTH IN mμ

particles of the HR-LT strain are also 180 μ long indicating again that the B particles of the two strains are probably morphologically identical. The LT particle, however, measured 100 μ in length, which is just over half the length of the B particle. The HR-LT strain of VSV therefore produces B particles similar to those of the wild type strain, and T particles which are about 60% longer than the ST particles.

2. RNA of B and LT Particles of the HR-LT Strain of VSV

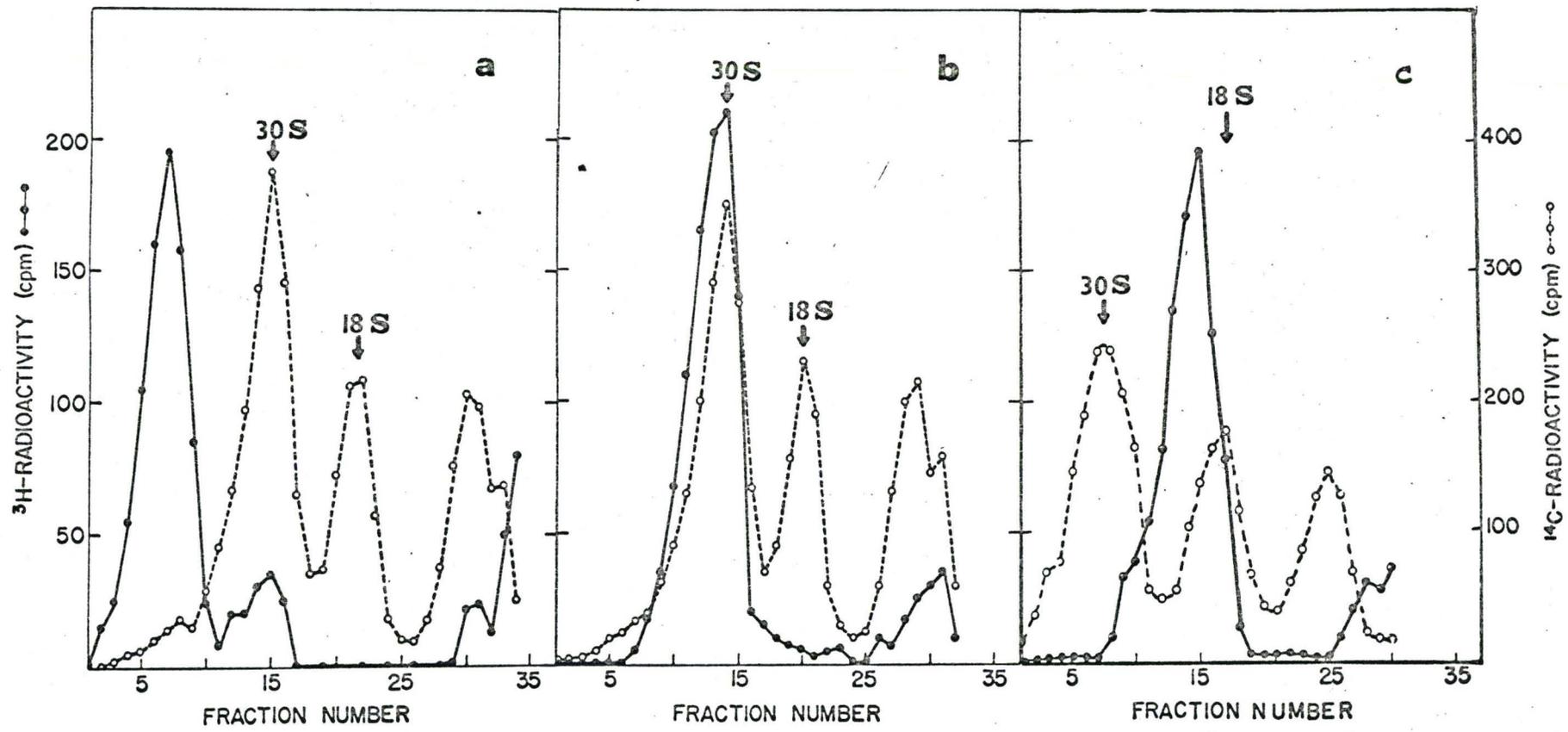
Since the LT particle is intermediate in length between the ST and B particles, the molecular weight of its RNA genome would be expected to be intermediate between the ST and B particle RNAs. RNA was extracted from sucrose gradient purified, ^3H -uridine labelled, B, LT and ST particles. Each extract, to which were added 5000 cpm of ^{14}C labelled ribosomal RNA was sedimented on a 5 to 20% sucrose gradient as outlined in the Methods. The gradients were then fractionated, and monitored for ^3H and ^{14}C radioactivity (fig. 4).

If cytoplasmic RNA species are assigned sedimentation coefficients of 30, 18, and 4S in the linear sucrose gradient, then the sedimentation coefficients of virion RNAs can be estimated. On this basis B particle RNA (fig. 5a) and ST particle RNA (fig. 5c) species sediment at 43S and 20S respectively. These values are in close agreement with those of Huang and Wagner (1966b). The LT particle RNA on the other hand (fig. 5b) has a sedimentation coefficient of 30 to 31S.

An estimate of the molecular weight of the LT particle RNA can be obtained from the sedimentation coefficients. For this operation the relationship $M = aS^b$ where M is molecular weight, S is the sedimentation

fig. 5 Sucrose gradient analysis of RNA from B (a), "long T" (b) and "short T" (c) particles.

RNA extracted from ^3H -uridine labelled gradient purified virions (fig. 1) with SDS (0.5%) was precipitated by the addition of 2 volumes of ethanol. The precipitates along with a small amount of ^{14}C labelled L cell ribosomal RNA serving as marker were centrifuged in 5 to 20% sucrose gradients in STE-SDS buffer. After centrifugation at 185,000 X g for 2 hours successive fractions were collected onto glass-fiber filter pads by dripping from the bottom of the gradient tubes. Plotted are ^3H counts in viral RNA (- ● - ● -) and ^{14}C counts in ribosomal and transfer RNA (- o - o -).



coefficients, and a and b are constants (Spirin 1963) must be assumed to hold. If the B and ST particle RNAs are taken to have molecular weights of 3.6×10^6 daltons and 1.1×10^6 daltons respectively (Nakai and Howatson 1968), the values for a and b can be calculated. The molecular weight of the LT particle RNA, therefore, falls between 1.7×10^6 and 2.0×10^6 daltons. This ratio of molecular weights (1.1:1.7:3.6) of the RNA of these particles is in good agreement with the actual lengths of these particles.

The long T particle is thus a distinct morphological entity produced by the HR-LT strain of VSV. Its length and the molecular weight of its RNA are intermediate between those of the ST and B particles.

II. VIRUS SPECIFIC RIBONUCLEOPROTEIN IN INFECTED CELLS

Significant amounts of virus specific RNA have been shown by Schaffer et al. (1968) and Stampfer et al. (1969) to be present in cells infected with VSV. A large amount of this RNA has been reported to have sedimentation coefficients of 43S and 19S even though little mature virus is present in VSV infected cells (Howatson and Whitmore 1962). It would therefore be of interest to know if these species of RNA were associated with some virus specific structure present in the infected cell.

1. ³H-Uridine Labelling of the Cytoplasmic Extract

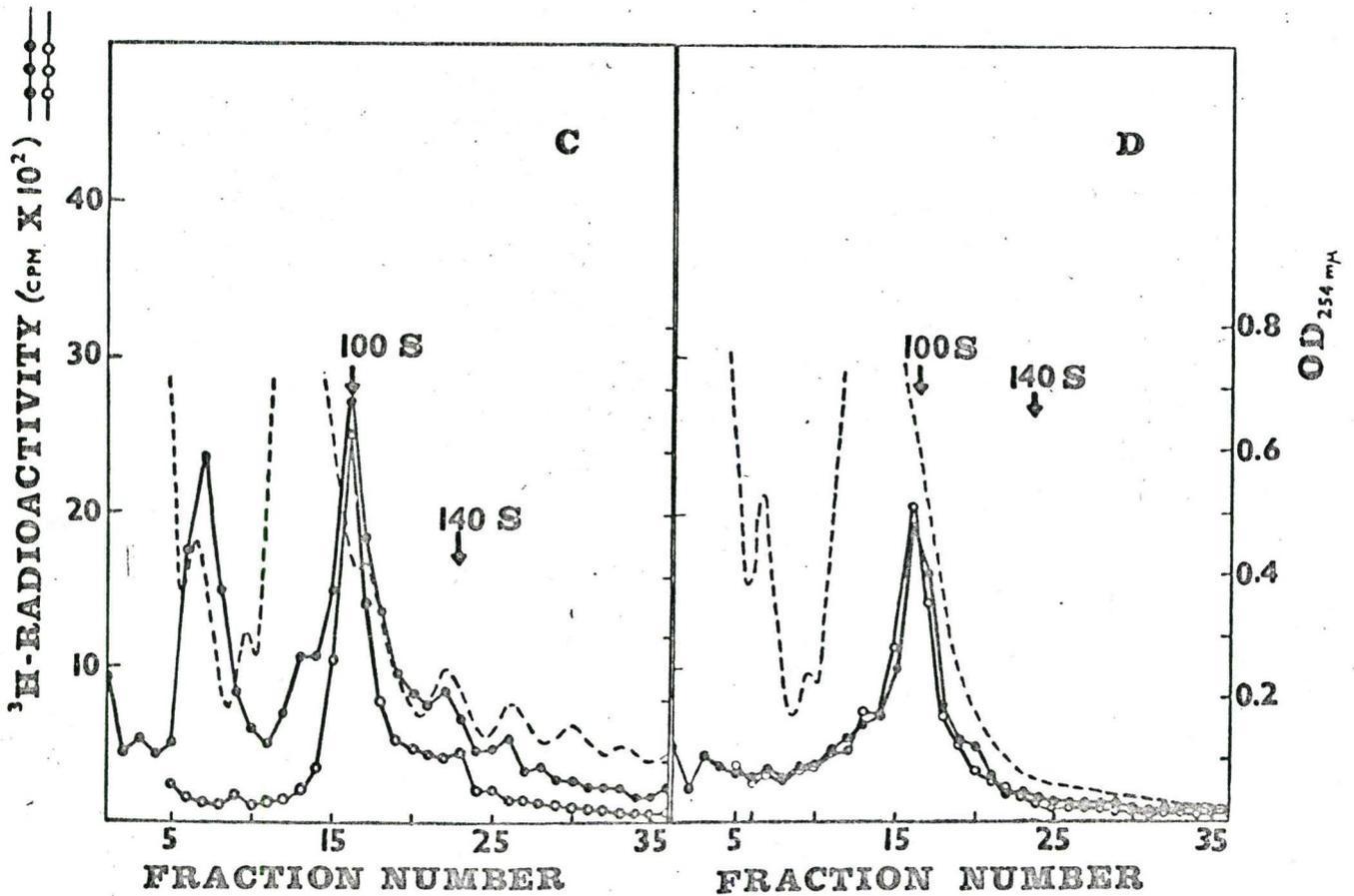
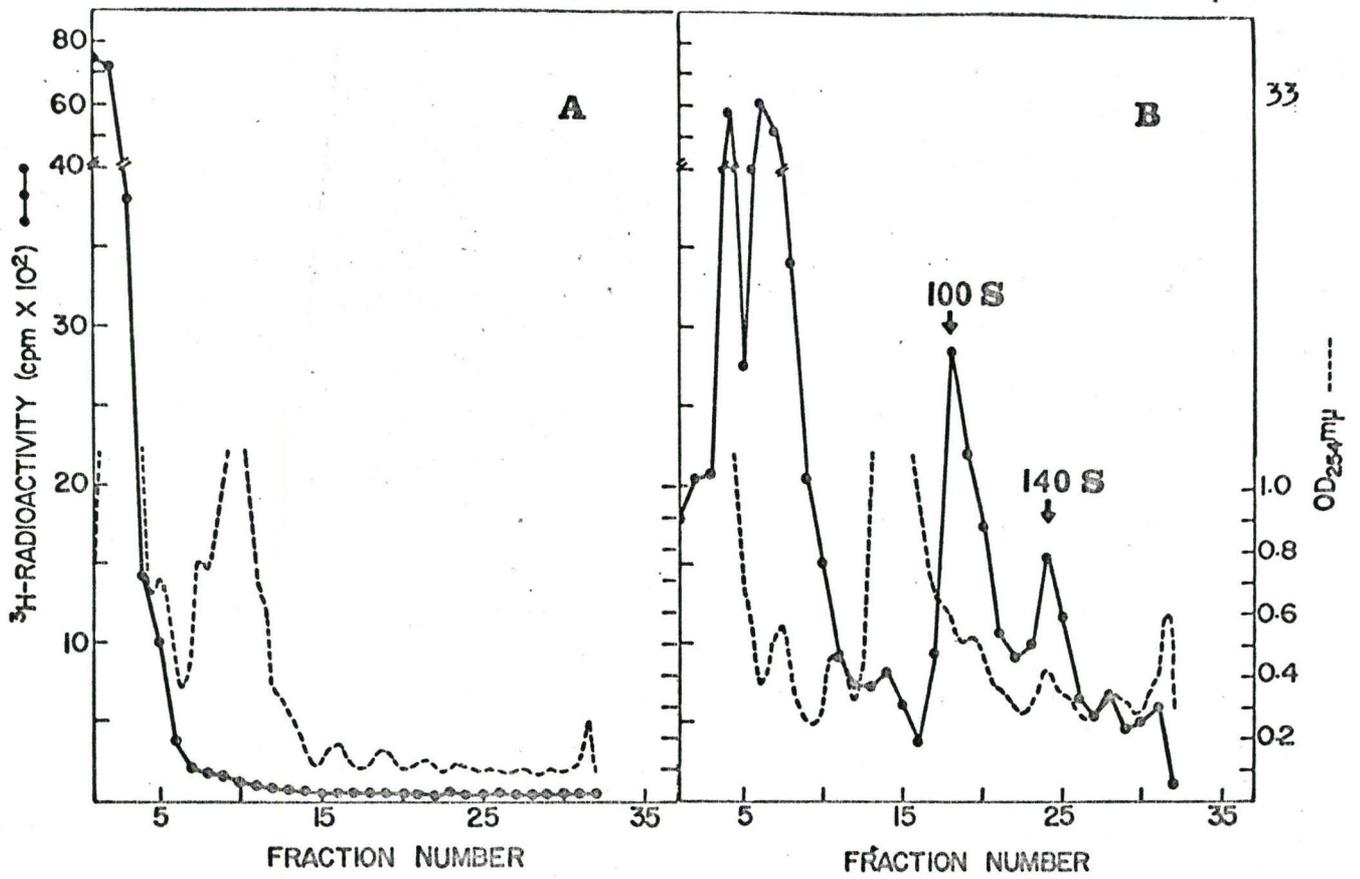
Cell cultures were infected with the HR-LT strain of VSV, treated with Act. D and labelled with ³H-uridine as outlined in Chapter 2. At 5 hours post infection the cells were harvested, homogenized, and their cytoplasmic extract sedimented on 15 to 30% sucrose gradient. Following centrifugation the gradients were fractionated, and monitored for absorbance (254 mμ) and radioactivity.

Figure 6a presents the results of a control, uninfected, but Act. D treated culture. In this case the cytoplasmic extract was centrifuged on a sucrose gradient for 2 hours at 58,000 X g, a shorter centrifugation than given to infected cell cytoplasmic extracts. The absorbance peak at fraction 10 is due to ribosomes whose sedimentation coefficient we have assumed to be 80S (Peterman 1964). To the right of the single ribosomes with greater sedimentation coefficients are absorbance peaks corresponding to the various polyribosome fractions. This optical density pattern serves as an internal standard for the sedimentation coefficients of other

fig. 6 Cytoplasmic Extracts of Infected and Uninfected Cells

Cytoplasmic extracts of cells infected or mock infected at M.O.I. of 50, treated with Act. D and labelled with ^3H -uridine were analyzed on 15 to 30% sucrose gradients in RSB⁺ at 58,000 X g for 2 hours (a) and 6 hours (b,c,d). The optical density (-----) at 254 m μ was continuously monitored as 1 ml. fractions were collected. Each fraction was precipitated onto filters with 5% TCA and its radioactivity determined.

(a) A mock infected culture, (b) Cytoplasmic extract of an infected culture, (c) Cytoplasmic extract of a second infected culture. Here the gradient fractions were split into two portions of which one was directly acid precipitated and its radioactivity determined (—●—●) while the other portion was treated with RNase (5 $\mu\text{gm/ml.}$) for 30 minutes then TCA precipitated (—○—○). (d) A duplicate of (c) except that prior to analysis on the gradient the extract was treated with 5 μgm of RNase at 40°C. The remaining procedure was as in (c) above.



cytoplasmic macromolecules.

Very little radioactivity (as represented by the solid line) is associated with structures greater than 40S in this cytoplasmic extract. Since functional messenger RNA (m-RNA) and cellular ribosomal RNA (r-RNA) would normally be found in the polyribosomes within 30 minutes of synthesis, this result indicates that Act. D has been effective in suppressing host cell RNA synthesis.

The radioactivity and absorbance along a gradient containing infected cytoplasmic material is shown in fig. 6b. In this gradient the single ribosomes are located at fraction 15 while 40S and 60S ribosomal subunits are resolved at fraction 7 and 11 respectively. It is of some interest that the dimers (110S), trimers (140S) and even tetramers (160S) of polyribosomes in the infected cells are each partially resolved into two fractions. The possible significance of this result will be considered in the Discussion. Looking at the radioactive virus-specific RNA in the gradient it is seen that while the radioactivity is present throughout the polyribosome region of the gradient there are distinct peaks of radioactivity at fractions corresponding to sedimentation coefficients of 40S, 100S and 140S.

The significance of the peaks of radioactivity at 100 and 140S becomes apparent when one considers that ribonucleoprotein (RNP) extracted from LT and B particles sediments at 100 and 140S respectively (Kang and Prevec 1969). The possibility, therefore, exists that these intracellular fractions may represent viral RNP present in the cytoplasmic extract. If so, we would expect RNA present in these fractions to be resistant to RNase

since Wagner et al. (1969b) showed that RNP from the virion was not RNase sensitive. Accordingly an infected cell cytoplasmic extract prepared as indicated above was split into two portions. One portion was treated with pancreatic RNase (5 $\mu\text{gm/ml.}$) and then both portions were sedimented through a sucrose gradient. After fractionation and monitoring for optical density at 254 m μ each sucrose gradient fraction was split into two equal portions. While one part of each fraction was immediately precipitated with TCA and assayed for radioactivity, the other was treated with pancreatic RNase (5 $\mu\text{gm/ml.}$) for 30 minutes at 37°C and then precipitated with TCA and assayed for radioactivity.

Treatment of each gradient fraction with RNase after centrifugation shows that while the RNA sedimenting at 40S and that associated with the polyribosomes are sensitive to RNase, the material at 100S and to some extent that at 140S is resistant to the enzyme (fig. 6c).

If the cytoplasmic extract was treated with RNase prior to centrifugation on the gradient, only the 100S material appears unaffected (fig. 6d). The fraction originally sedimenting at 140S is not present in this gradient suggesting that while RNA in this fraction is protected from extensive degradation by RNase, the 140S fraction can be converted to lower molecular weight material by the enzyme. The breakdown of polyribosomes as evidenced by the optical density profile in this gradient ensures that the RNase treatment was effective in breaking single-stranded m-RNA.

In summary: the newly synthesized virus specific RNA sediments in an RNase sensitive form both at 40S and associated with the polyribosome region of the cytoplasmic extract. In addition, viral RNA is present in

an RNase resistant form in structures sedimenting at 100S and 140S.

2. RNA of the Cytoplasmic Fractions

As it had previously been shown by Schaffer et al. (1968) and Stampfer et al. (1969), at least three separable RNA species with sedimentation coefficients of 43S, 30S and 15S should be present in infected cells. In order to study the role of these viral RNA species, it was necessary to establish their association with each of the intracellular structures described above.

The sucrose gradient fractions containing the particular RNA structures (i.e. 40S, 80S, 100S, and 140S) were separately collected and their RNA was extracted and analysed on a sucrose gradient, as outlined in the Methods.

If, as suggested previously, the 100S and 140S regions of the cytoplasmic gradients contain structures similar to the ribonucleoprotein (RNP) from the respective LT and B particles, these regions should contain 30S and 43S RNA which is characteristic of these virions. As can be seen in fig. 7, only the 140S region contained 43S RNA which is found in the cell cytoplasm, and the 100S region contained a large proportion of the 30S RNA. In addition, all of the cytoplasmic fractions examined contained a 15S species of RNA and with the exception of the 40S cytoplasmic region all fractions also contained a 30S RNA species. The finding that 15 and 30S RNA were associated with cellular polyribosomes suggested the possibility that these species may serve as virus specific m-RNA.

The fact that 15S RNA sediments as an RNase sensitive fraction with a sedimentation coefficient of 40S in a cytoplasmic extract could be due

fig. 7 Analysis of the virus specific RNA found in selected fractions of the infected cell cytoplasmic extract.

RNA present in ^3H -uridine containing fractions of the 40S region (a), 80S region (b), 100S region (c), and 140S region (d) of a cytoplasmic extract identical to that seen in fig. 6b was extracted with SDS and precipitated with ethanol. The RNA precipitates were dissolved in STE-SDS buffer and analysed on 5 to 20% sucrose gradients in STE-SDS buffer. The optical density 260 m μ (-----) and radioactivity (—●—●) of each fraction are plotted.

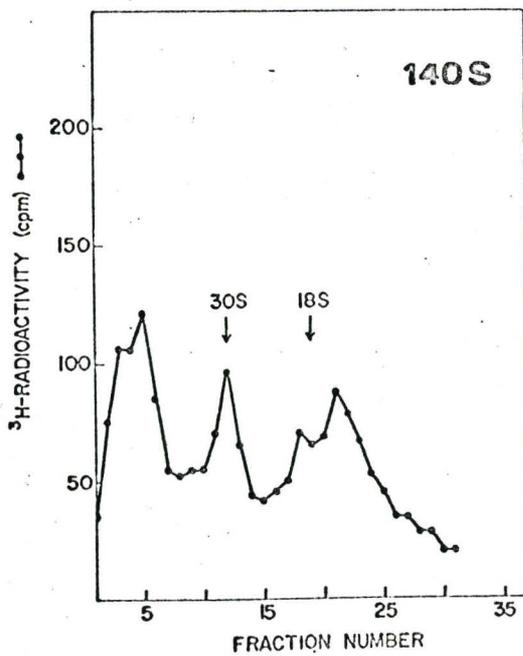
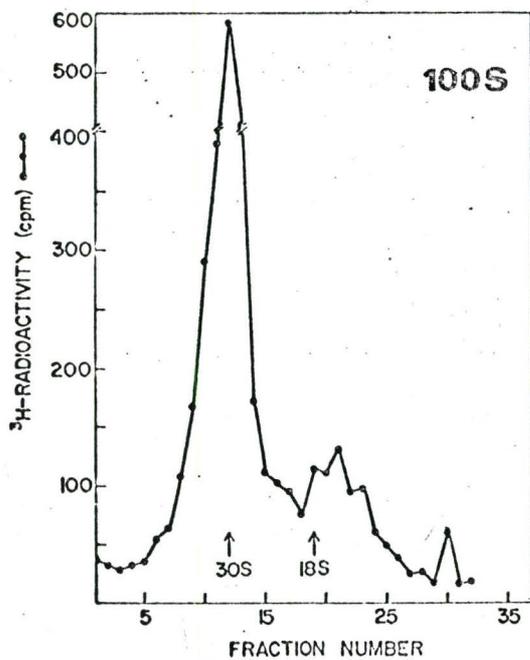
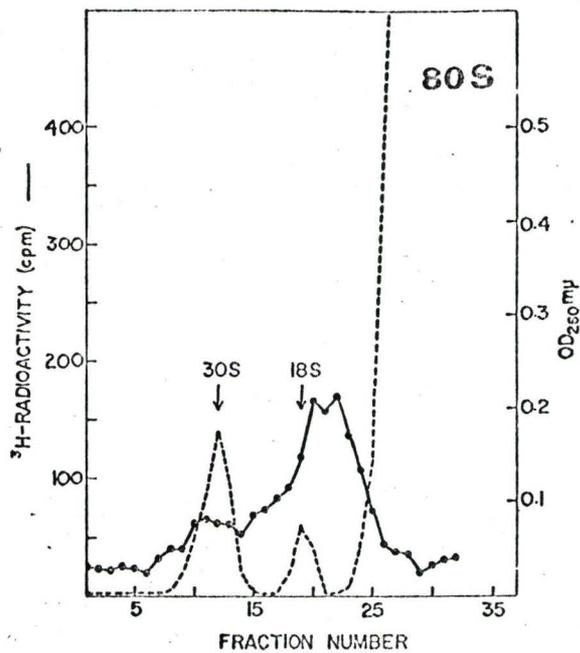
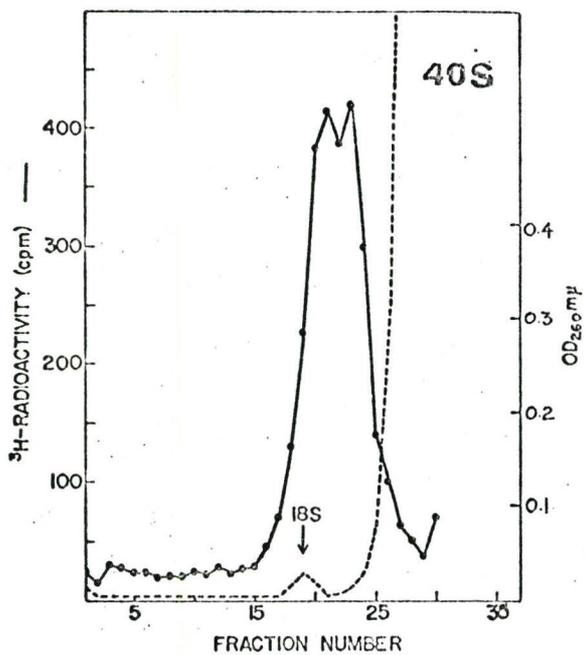
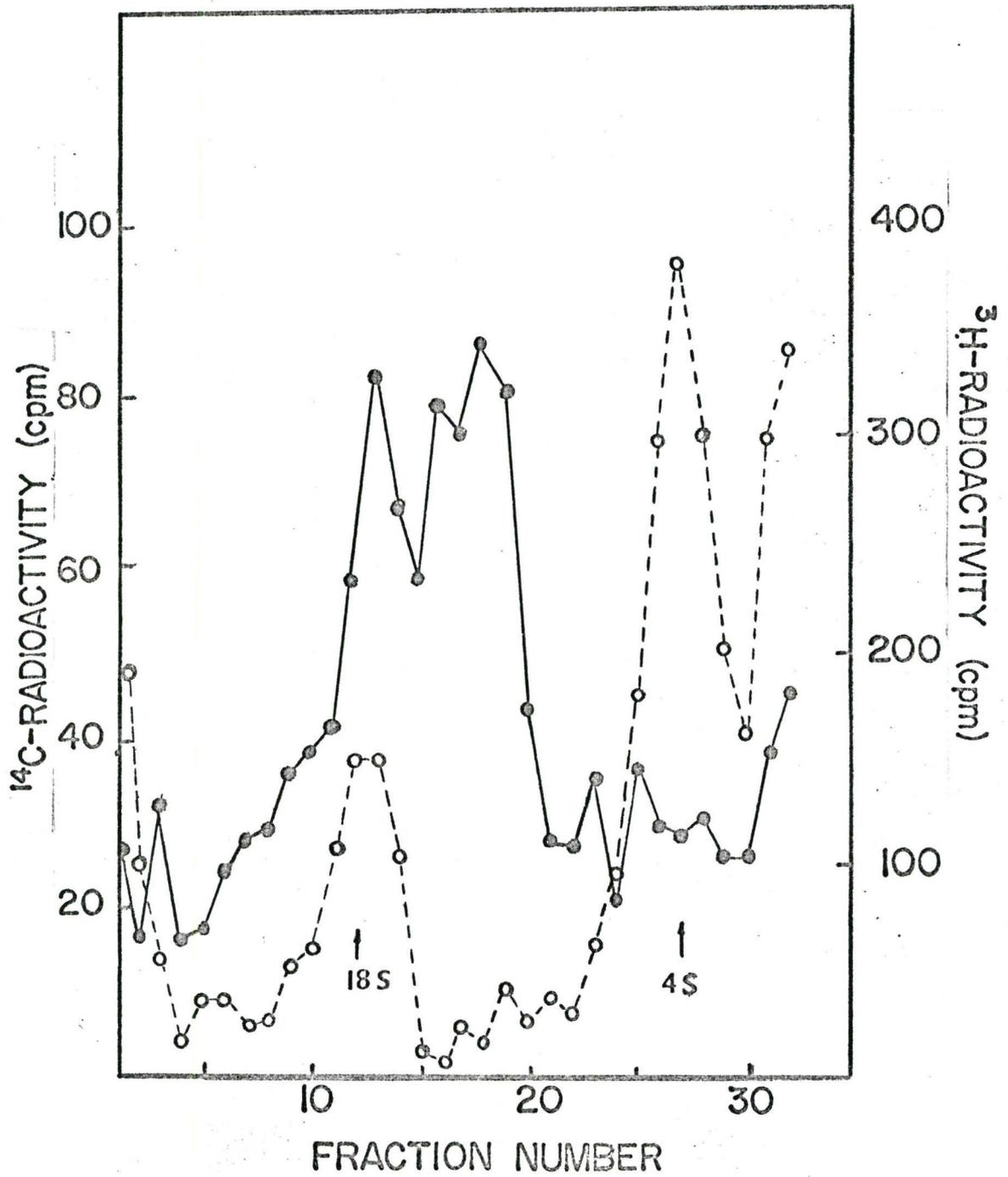


fig. 8 Analysis of 15S polyribosomal RNA.

RNA was extracted with SDS from the total polyribosomal fraction of a sucrose gradient (containing the cytoplasmic extract of infected Act. D treated, ^3H -uridine labelled cells) as shown in fig. 6b. After it was precipitated out of solution with ethanol, the RNA was redissolved in STE-SDS buffer, layered on a 5 to 20% STE-SDS sucrose gradient together with ^{14}C labelled ribosomal RNA to serve as marker, and centrifuged at 185,000 X g for 4 hours. The ^3H (—○—) and ^{14}C (—●—) radioactivity of successive fractions of the gradient were determined.



to the association of this RNA with 40S ribosomal subunits. Alternatively this may be due to the effect of the cytoplasmic extract which has been shown by Girard and Baltimore (1966) to increase the sedimentation coefficient of single-stranded RNA. In all of the graphs in fig. 7 the 15S RNA appears to be a rather heterogeneous species. From the molecular weights of the major structural proteins of VSV which are approximately 34,000 daltons, 60,000 daltons, and 81,000 daltons (Kang and Prevec 1969, Wagner et al. 1969a), it is possible to calculate the probable molecular weight and hence the sedimentation coefficient of individual m-RNA molecules required to code for these proteins. On this basis we would expect m-RNA molecules with sedimentation coefficients between 12S and 19S. When RNA extracted from the cytoplasmic fraction of infected cells was analysed by lengthy centrifugation (4 hrs. at 185,000 X g) the results shown in fig. 8 were obtained. As seen in this figure the 15S RNA may indeed consist of three distinguishable peaks with sedimentation coefficients of 18S, 15S, and 13S.

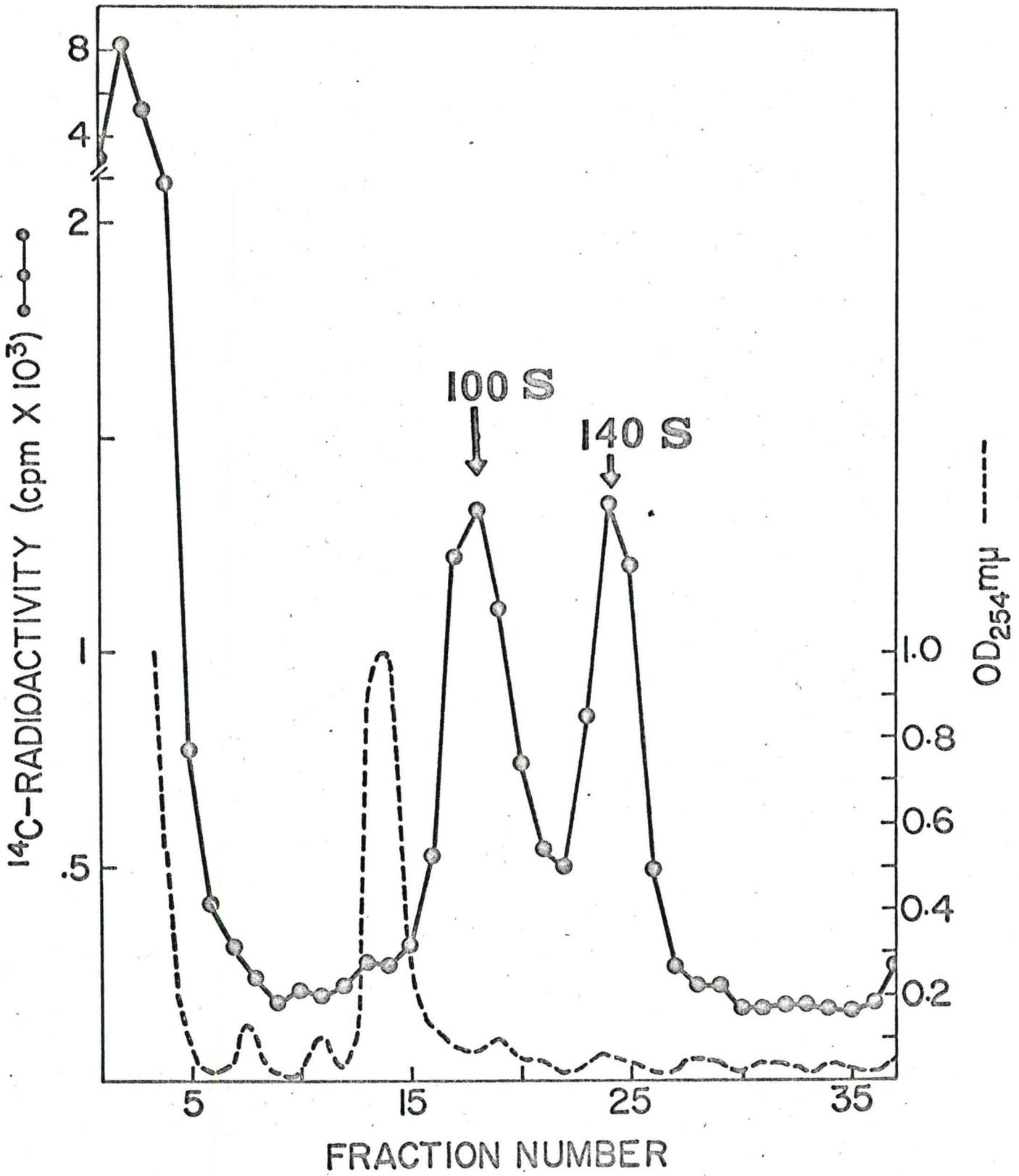
3. ¹⁴C-Amino Acid Labelled Cytoplasmic Extract

Evidence presented in the last two sections has indicated that the 100 and 140S fractions of the cytoplasmic extract may contain virus specific RNP. If so, these fractions should contain large amounts of newly synthesized virus specific protein.

To investigate this possibility a cytoplasmic extract of virus infected cells, treated with Act. D and labelled with ¹⁴C-amino acids was sedimented on a sucrose gradient as described in the Methods. Figure 9, representing the distribution of the cytoplasmic extract on a sucrose gradient, demonstrates that a relatively large quantity of newly synthesized protein identified by

fig. 9 ^{14}C -amino acid labelling of cytoplasmic fractions from infected cells.

Approximately 10^8 cells, infected with the HR-LT strain of VSV were treated with Act. D. At 3 hours post infection the cells were removed from the medium, resuspended in MEM containing 1/20th the normal amino acid concentration and labelled with a mixture of ^{14}C amino acids (0.2 $\mu\text{c}/\text{ml}$.). The cytoplasmic fraction was extracted from the cells at 5 hours post infection, and analyzed on a sucrose gradient as described in fig. 6b. The optical density along the gradient (---) and radioactivity of each fraction (●—●—●) are shown.



^{14}C incorporation into TCA insoluble material is indeed associated with the 100 and 140S fractions. As well, a considerable portion of the newly synthesized protein remains on the top of the gradient. Since in mock infected cells treated as above, no peaks of radioactivity are present beyond 80S, the above result indicates that the 100 and 140S fractions contain newly synthesized viral protein, most likely in the form of RNP.

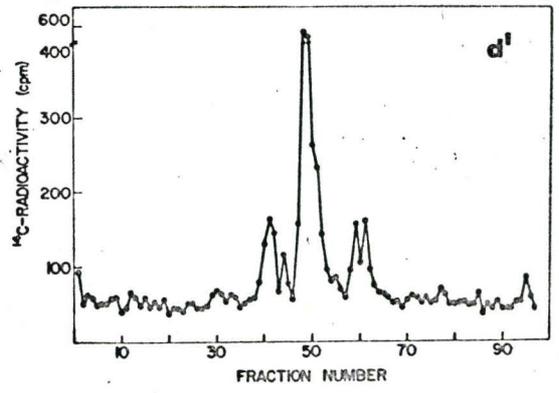
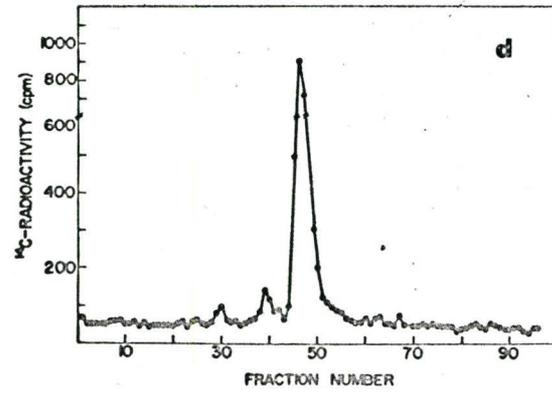
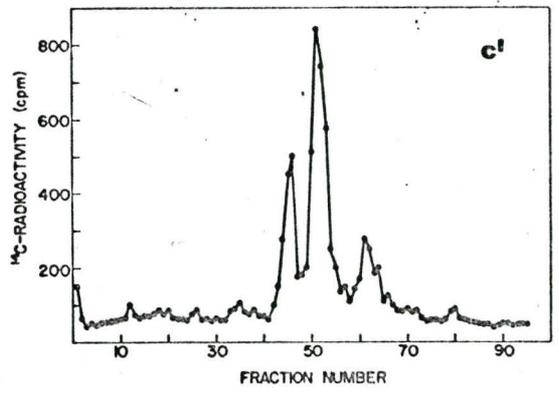
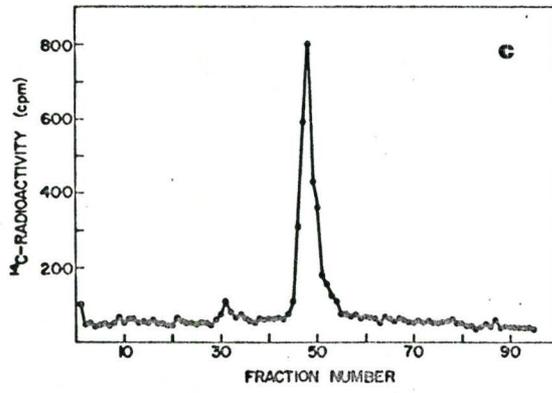
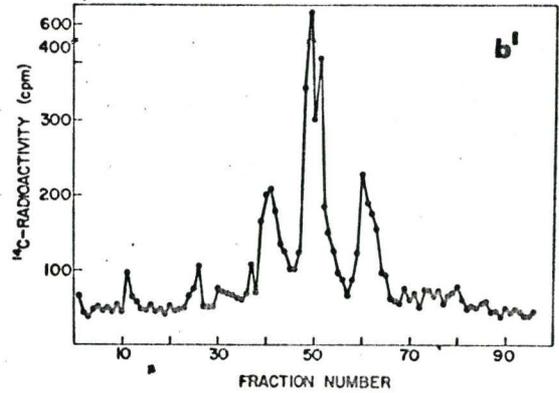
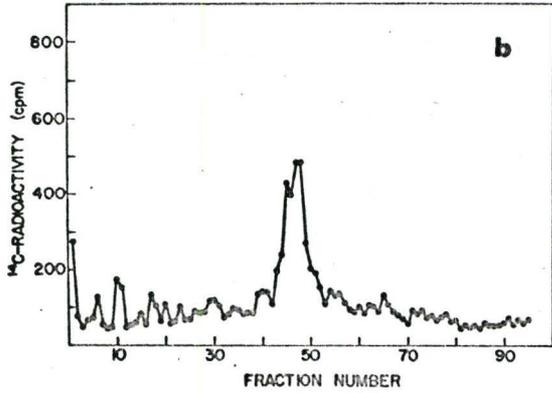
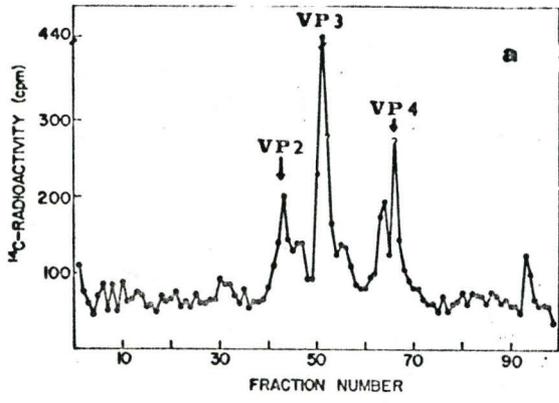
4. Characterization of the Virus-Specific Protein Present in the Infected Cell Cytoplasmic Extract

The newly synthesized virus specific protein present in the 100 and 140S cytoplasmic fractions of virus infected cells could be identified as a component of the RNP by polyacrylamide gel electrophoresis. Kang and Prevec (1969) have shown that the virus contains 4 distinct structural proteins. Of these, virus protein 3 (VP-3) is the only protein present in the viral RNP. An attempt was therefore made to identify the protein present in the 100 and 140S regions and the low molecular weight material present in the top five fractions of the sucrose gradients containing extracts of VSV infected ^{14}C -amino acid labelled cells, were individually pooled and concentrated after overnight dialysis against RSB⁺. The protein of each preparation was denatured and analysed on polyacrylamide gels as outlined in the Methods.

The distribution of the protein on the gels is presented in fig. 10. It may immediately be seen that the protein present in the 100S (fig. 10c) and 140S (fig. 10d) fractions migrates as a single entity. When these proteins were coelectrophoresed with ^{14}C labelled viral structural protein (fig. 10c' and 10d' respectively) only VP-3 was found to exhibit an increase in radio-

fig. 10 Polyacrylamide gel analysis of ^{14}C -amino acid labelled fractions from infected cell cytoplasm.

Highly radioactive fractions from the top component (free protein), the 100S region and the 140S region of a gradient identical to that in fig. 9 were separately pooled. After the removal of the sucrose by dialysis, pooled material was concentrated by flash evaporation. After treating the preparations with urea, acetic acid, SDS, and mercaptoethanol samples containing 40,000 cpm were put on acrylamide gels with or without an additional 40,000 cpm of ^{14}C -labelled marker viral proteins. The gels were run for 15 hours at 3 volts/cm, sliced and the radioactivity of each slice determined. Virus marker alone (a), protein in the low molecular weight component (b), and with virus marker (b'), protein from the 100S region (c), and with marker (c'), and protein from the 140S region (d), and with virus marker (d') are plotted.



activity. Hence the virus specific protein present in the 100 and 140S fractions migrates as protein characteristic of RNP, namely VP-3.

Because counting efficiency was different for each slice the amount of radioactivity in each gel may vary considerably so that only the relative number of counts per peak should be considered.

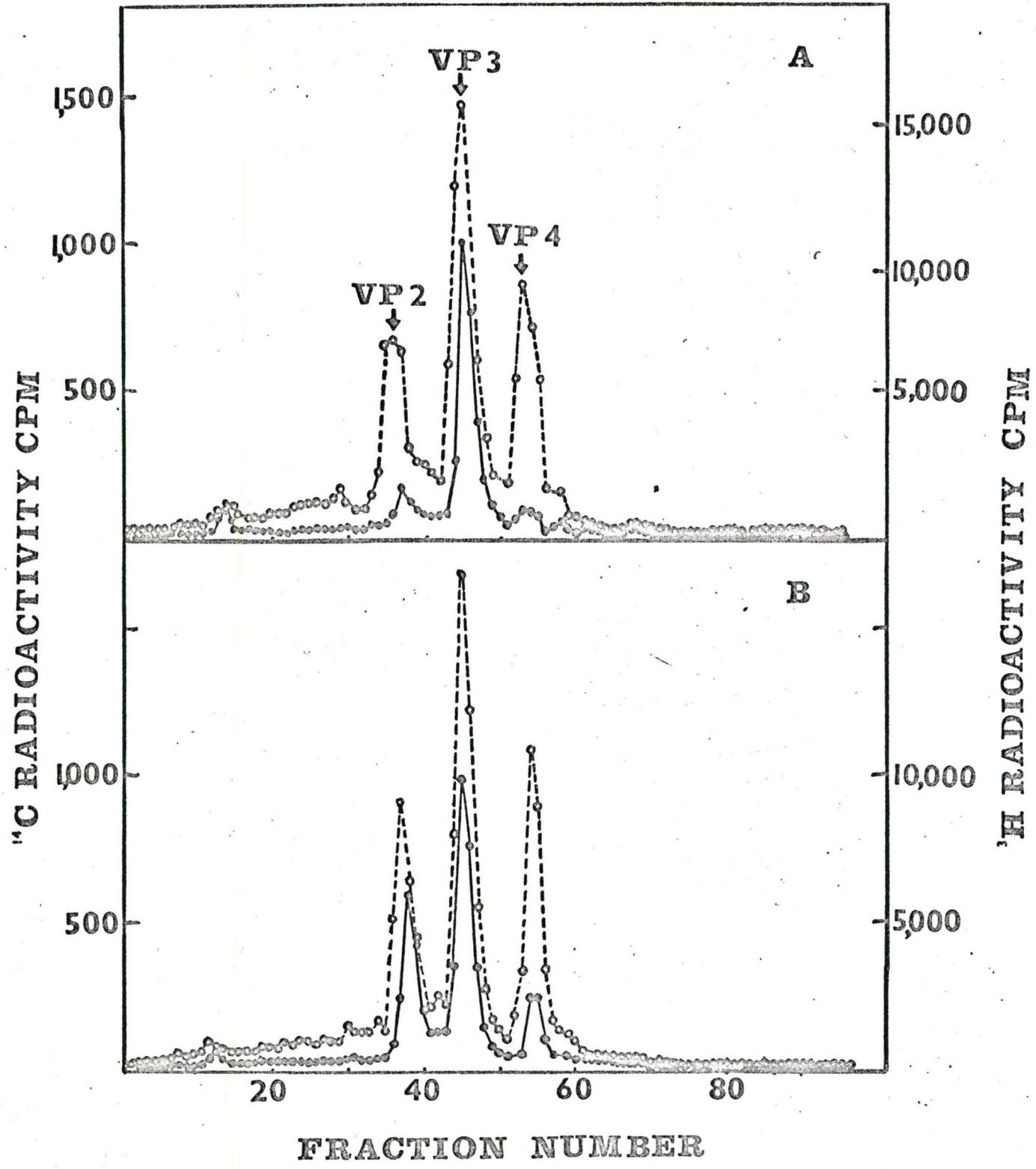
When the proteins of the low molecular weight component of the above cytoplasmic extract were examined by electrophoresis, the results shown in fig. 10b were obtained. Here again only one peak appears. If this material is coelectrophoresed with labelled viral structural protein as shown in fig. 10b', only VP-3 shows an increase in radioactivity. It therefore appears that VP-3 is the only protein present in significant amounts in the cytoplasmic extract of infected cells.

This finding raises the question about the intracellular location of VP-2 and VP-4 which, as was shown in fig. 10a are present in substantial quantities in the virus. Accordingly an experiment was performed in which Act. D pretreated cells were infected and labelled with ^{14}C -amino acids. On harvesting, the cells were split into two equal portions. The cytoplasmic extract of one portion was treated with virus-disrupting-buffer, while the whole cell pellet of the other portion was treated with the SDS-MCE solution and sonicated as outlined in Chapter 2. The proteins of both portions were analysed on acrylamide gels.

The results shown in fig. 11 demonstrate that while the cytoplasmic extract contains chiefly VP-3, all the major viral proteins are present in the whole cell in a ratio similar to that in which they are present in the virus. This result suggests that although all the viral structural proteins

fig. 11 Comparison of the viral protein in the total cell extract and in the cytoplasmic fraction extract.

Cells pretreated with Act. D (0.5 ug/ml.) for 8 to 12 hours were infected and labelled with ^{14}C -amino acids. Prior to harvesting, the culture was split into two portions. The cytoplasmic extract of one portion was treated with virus disrupting buffer, while the cell pellet of the other portion was treated with SDS and mercaptoethanol and sonicated to reduce viscosity. Both extracts were dialyzed overnight against SDS, urea and mercaptoethanol as described in the Methods. After addition of ^3H labelled viral proteins as markers the above preparations were examined on polyacrylamide gels. (A) cytoplasmic extract; (B) total cell extract. ^3H -marker proteins (—○—○—○—); ^{14}C proteins (—●—●—●—).



are present in the infected cell only VP-3 is found free in the cytoplasm. Both VP-2 and VP-4 seem to be associated with some structure, most likely membrane, which is removed in the preparation of the cytoplasmic extract.

5. Ribonucleoprotein in Cells Infected with IND-ST

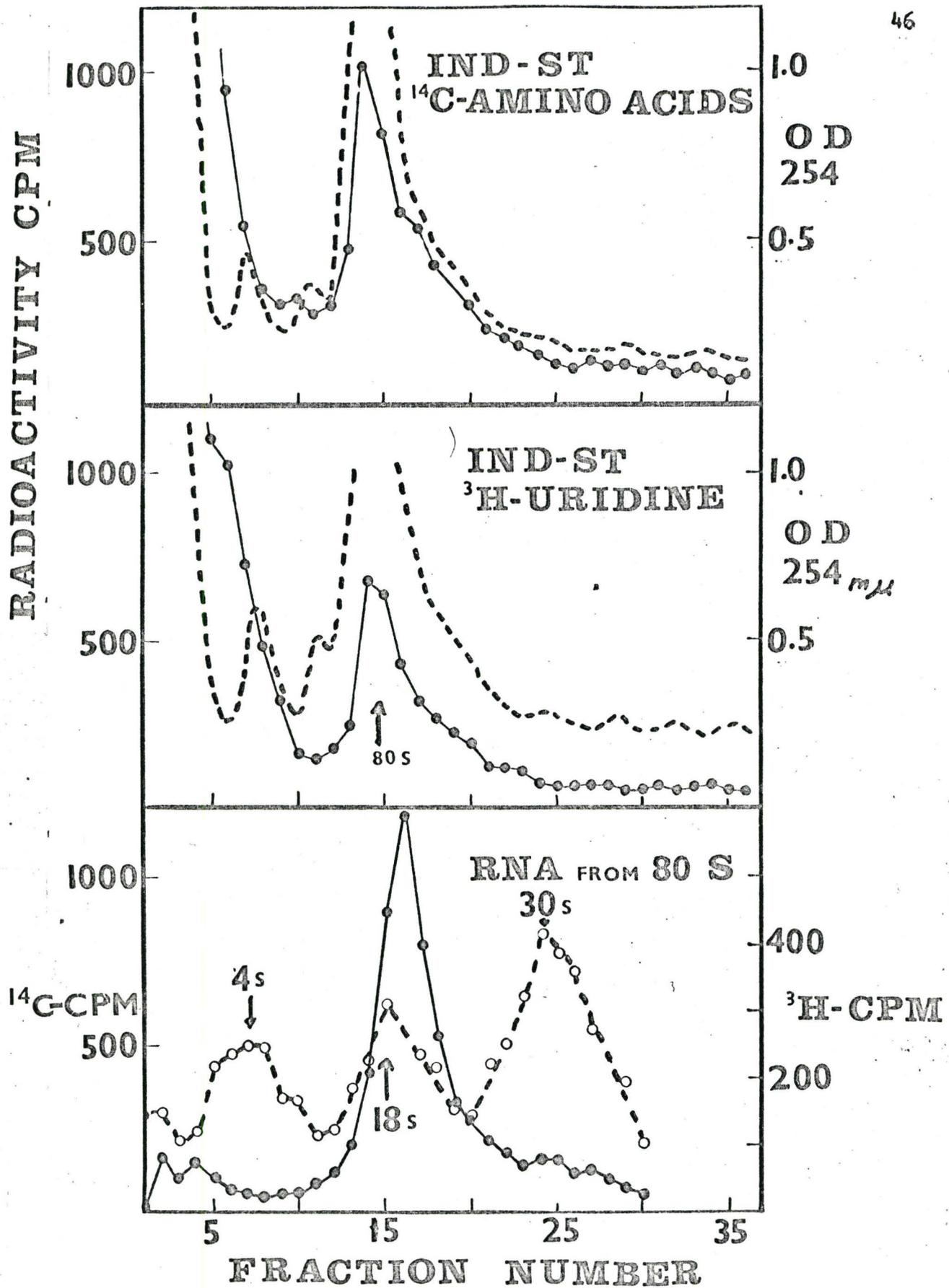
If virion RNP accumulates within infected cells its sedimentation coefficient should be characteristic of the types of virions being produced in infection. Thus, RNP produced in cells infected with HR-LT strain has sedimentation coefficients of 100S and 140S. Similarly cells infected with the IND-ST strain of VSV might be expected to contain RNP characteristic of short T particles which are the major defective particles produced by this strain.

An experiment was therefore performed, as described in section 3 above, using IND-ST as the infecting virus. The results shown in fig. 12 illustrate that the 80S region of the cytoplasmic extract now contains most of the newly synthesized RNA and protein. Little newly synthesized material is present in the 140S fraction since infection was carried out with a virus stock containing many T particles, hence not favouring the production of B particles.

The RNA of a portion of the 80S region of the cytoplasmic extract labelled with ^3H -uridine was examined by sedimentation on a 5 to 20% sucrose gradient. The RNA sedimented at 20S which is also the sedimentation coefficient of RNA extracted from the ST virion. Therefore the size of the RNP produced in the infected cell is characteristic of the type of particles being produced by the infected cells.

fig. 12 Cytoplasmic extracts from cells infected with IND-ST strain of VSV and a gradient analysis of the RNA of the 80S region of the cytoplasmic extract.

Two identical cultures of about 5×10^7 cells were separately infected with the IND-ST strain of VSV at M.O.I. of 50 and treated with Act. D at 30 min. post infection. The cells of one culture were resuspended in amino acid deficient medium at 3 hours post infection as described in fig. 9 and labelled for 1.5 hours with ^{14}C -amino acids (0.2 $\mu\text{c/ml.}$). The second culture was labelled from 3 to 5 hours with ^3H -uridine. Separate cytoplasmic extracts of the above cultures were layered on sucrose gradients which were centrifuged at $81,000 \times g$ for 4 hours, and fractionated after they were monitored for optical density (---). A portion of each fraction was precipitated with ice cold TCA and the radioactivity shown in the top two panels determined. The remaining material from the 80S region of the ^3H -uridine labelled gradient was pooled, treated with SDS and the RNA present precipitated with ethanol. This was analyzed on a STE-SDS sucrose gradient with ^{14}C ribosomal RNA acting as marker. The ^3H (-e-e-e-) and ^{14}C (-o-o-o-) radioactivity were determined from successive fractions of the gradient are shown in the bottom panel.



6. Electron Microscopic Studies of the Cytoplasmic Fractions

This technique afforded the most conclusive evidence that virus specific RNP is present in the 100S and 140S cytoplasmic fractions. Electronmicrographs of negatively stained samples of the 100 and 140S cytoplasmic fractions are shown in fig. 13a and 13b respectively. The wavy ribbon-like strand running the length of each micrograph is very similar to the RNP seen by Nakai and Howatson in disrupted virions. The round granular objects present throughout the two photographs are ribosomes. It was found that while the 100S fraction contained mainly single ribosomes and dimers, the 140S fraction contained predominantly dimers and trimers. This data confirms that the 100S and 140S fractions contain virus specific RNP and that the optical density profile from which these sedimentation coefficients were derived was correctly interpreted.

To estimate the length of the RNP present in each of the above fractions six strands of RNP from 100S and 140S fraction electronmicrographs similar to those shown above were measured. The lengths of the 100S fraction RNP ranged between 1.6 to 2.1 μ while the lengths of the 140S fraction RNP were from 3.3 to 4.2 μ . Though these lengths deviate by a considerable amount they were nevertheless close to those one would expect for lengths of RNP from disrupted virions.

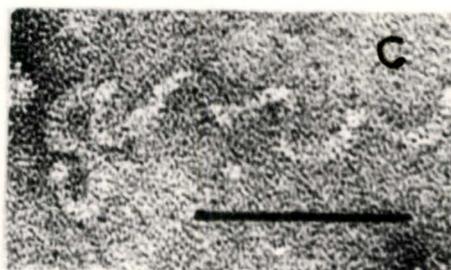
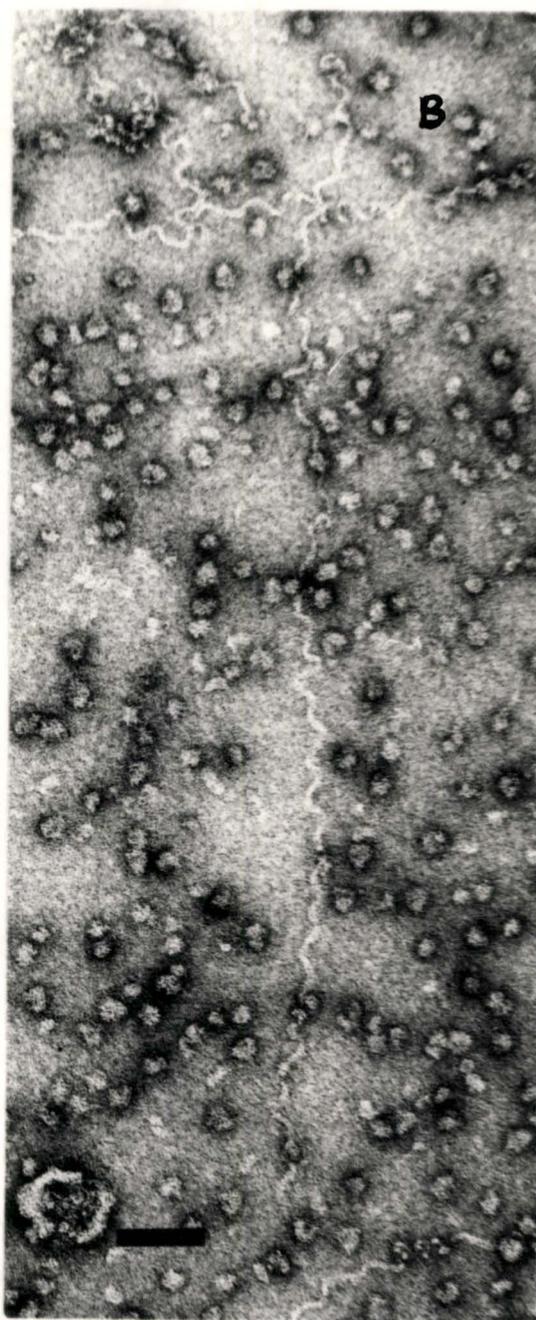
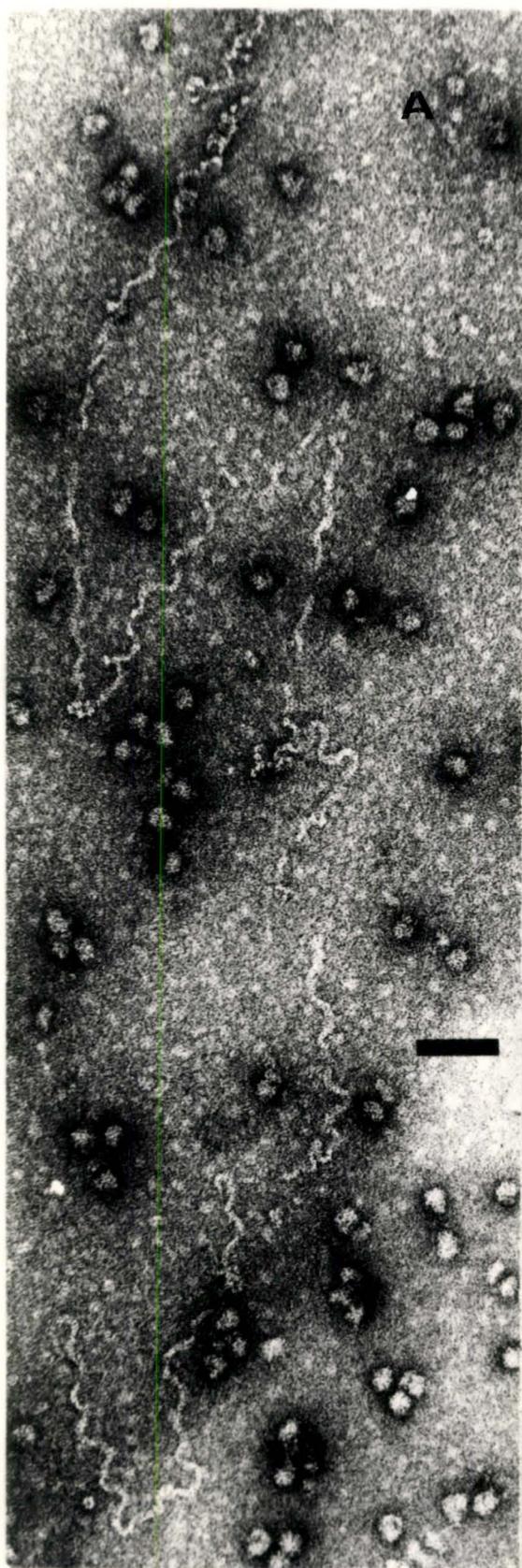
From all the evidence accumulated above, a form of RNP equivalent to that present in mature virions exists in virus infected cells.

fig. 13 Electron micrograph of material present in the 100S (A) and 140S (B) regions of the cytoplasmic extract which has been sedimented on a sucrose gradient. In C is shown a highly magnified portion of the above RNP structure.

Mag. of A and B X103,000

Mag. of C X250,000

Bar represents 0.1 μ



III. EFFECTS OF INTERFERENCE ON RNP

1. Function of RNP in the Infected Cell

The similarity of the intracellular RNP to that present in the mature virion suggests that it may act as a precursor to the virus. To examine this possibility a study of the infected cell RNP in this context was performed.

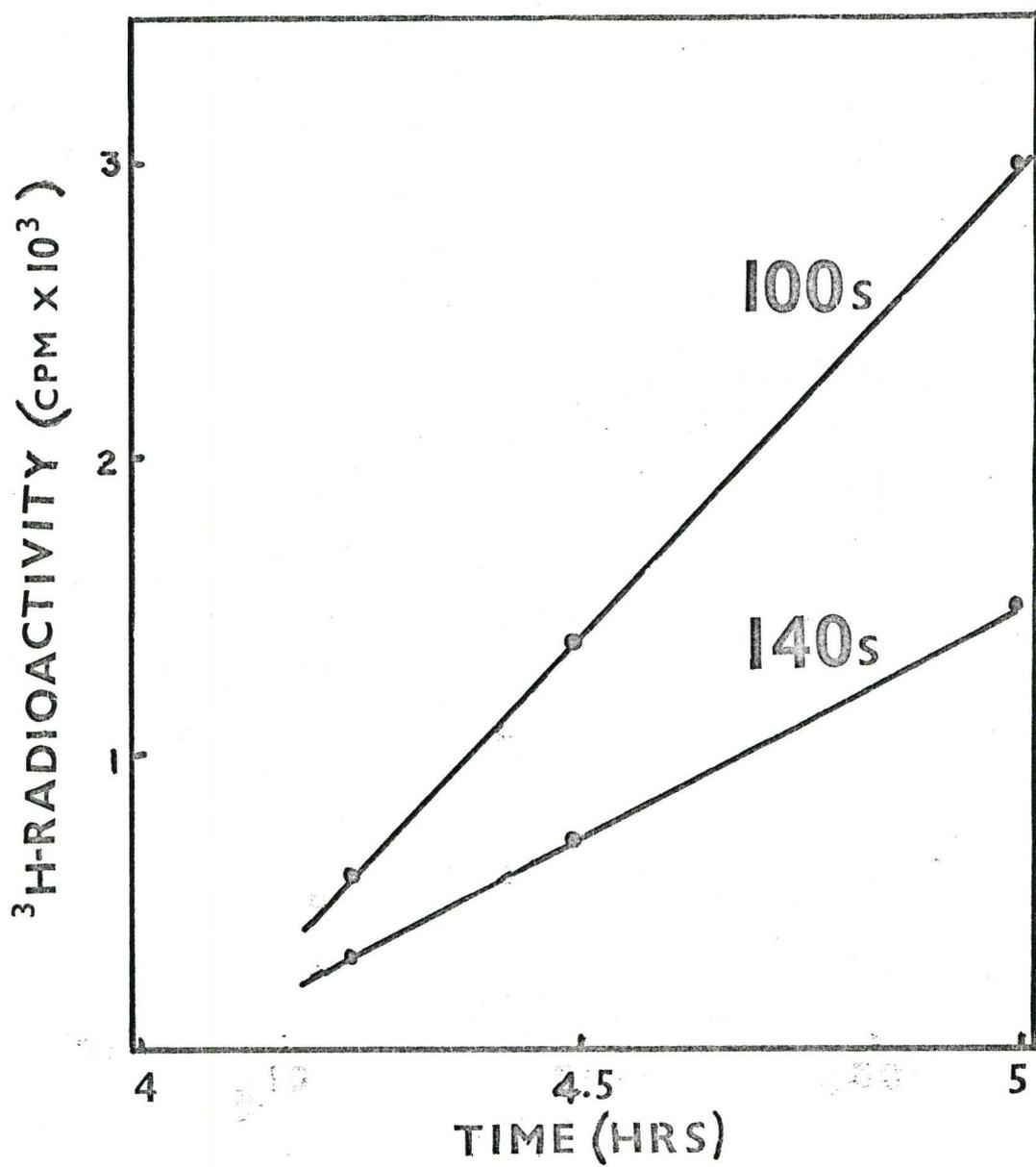
(a) Source of RNP. If the pool of RNP present in infected cells acted as a precursor of the virus, then labelling of the cells with ^3H -uridine at a period of high virus production (4-5 hours) should result in the incorporation of the ^3H -uridine until the precursor pool of RNP was saturated. At this point an equilibrium would exist between label entering the pool and leaving it to form mature virus.

Accordingly an infected cell culture was labelled with ^3H -uridine as indicated in the Methods. At 15, 30, and 60 minutes after labelling aliquots were withdrawn from the infected culture and rapidly chilled. The cytoplasmic extracts of these preparations were then analysed on sucrose gradients. For each gradient the radioactivity was summed over the 100 and 140S regions and plotted against the time of labelling.

Figure 14 shows that while both the 100 and 140S cytoplasmic fractions incorporate ^3H -uridine at similar rates, these rates do not appear to change as the labelling time continues. Since no plateau is indicated by these curves it appears that the 100 and 140S pools of RNP do not become saturated, but continued to incorporate ^3H -uridine at a constant rate.

fig. 14 Kinetics of ^3H -uridine incorporation into the 100 and 140S fractions of VSV infected cells.

Some 1.5×10^8 cells were infected with the HR-LT strain of VSV. Act. D (2 $\mu\text{gm/ml.}$) was added at $\frac{1}{2}$ hour and ^3H -uridine at $3\frac{1}{2}$ hours post infection. Equal samples were withdrawn from the culture at $4\frac{1}{4}$, $4\frac{1}{2}$ and 5 hours post infection, cytoplasmic extracts prepared and analysed on 15 to 30% RSB⁺ sucrose gradients. The radioactivity of the 100S and 140S regions was computed, normalized to the optical density in the extract and plotted.



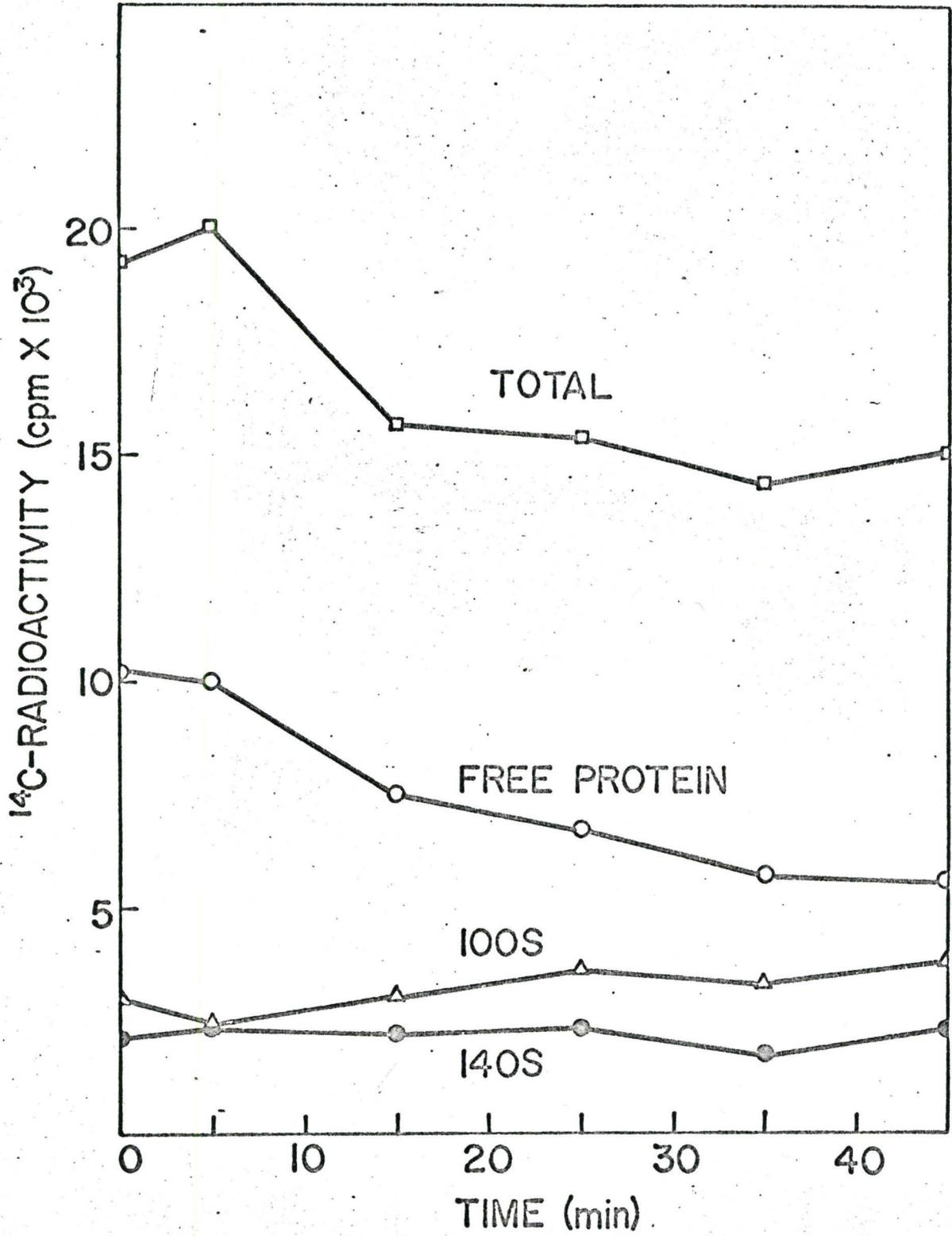
(c) Loss of RNP by Infected Cells. Since VSV leaves the cell by continuous budding through the membrane (Howatson and Whitmore 1962), then if RNP serves as a precursor to the virus, it should be possible by a pulse chase experiment to demonstrate the loss of RNP from the cell. Infected cells, at 4 hours post infection were therefore pulse labelled with ^{14}C -amino acids for 10 minutes and then transferred into medium containing a five fold concentration of non-radioactive amino acids which served as a chase for the ^{14}C -amino acids. Aliquots removed from this culture at 0, 5, 15, 25, 35, and 45 minutes after chase medium was added were chilled in ice cold PBS and their cytoplasmic extracts were analysed on sucrose gradients. Radioactivity totals of the 100S, 140S and low molecular weight region as well as of the whole gradient were obtained for each aliquot, normalized to the optical density and plotted against time of chase.

The radioactivity of the 100 and 140S fractions showed no decrease with length of chase (fig. 15). The only loss in radioactivity (which amounted to about 25% of the total) was exhibited by the low molecular weight cytoplasmic component. This material could have entered the RNP and hence accounted for the lack of RNP loss. However, since RNP had incorporated label so rapidly, and did not show any loss but rather a gain of radioactivity during a chase time four times the length of the pulse, it appears very unlikely that these RNP particles are precursors to the mature virus.

This evidence would suggest that the RNP present in the 100 and 140S fractions is a byproduct of virus assembly. Since all experiments so far were conducted under conditions which favoured viral interference,

fig. 15 Kinetics of the labelled viral proteins in the infected cell cytoplasm.

About 3×10^8 cells, infected with HR-LT strain of VSV and treated with Act. D were resuspended in low amino acid medium as described in fig. 9. The culture was resuspended in the low amino acid medium and ^{14}C -amino acids ($0.2 \mu\text{c/ml.}$) were added after 30 minutes. After 10 minutes of labelling an equal volume of MEM containing 10 times the amino acid concentration of normal MEM was added as a chase. At the times indicated on the graph, after the addition of the chasing amino acids, equal samples were withdrawn from the culture and cytoplasmic extracts prepared and analysed on sucrose gradients. The radioactivity in free protein, 100S and 140S region of the gradients for each time point was computed and normalized to the optical density of the extract. The normalized counts for each region and total counts in each gradient were plotted.



it was important to examine the cytoplasmic extract of cells infected under conditions which did not favour viral interference.

2. Conditions of Minimal Interference

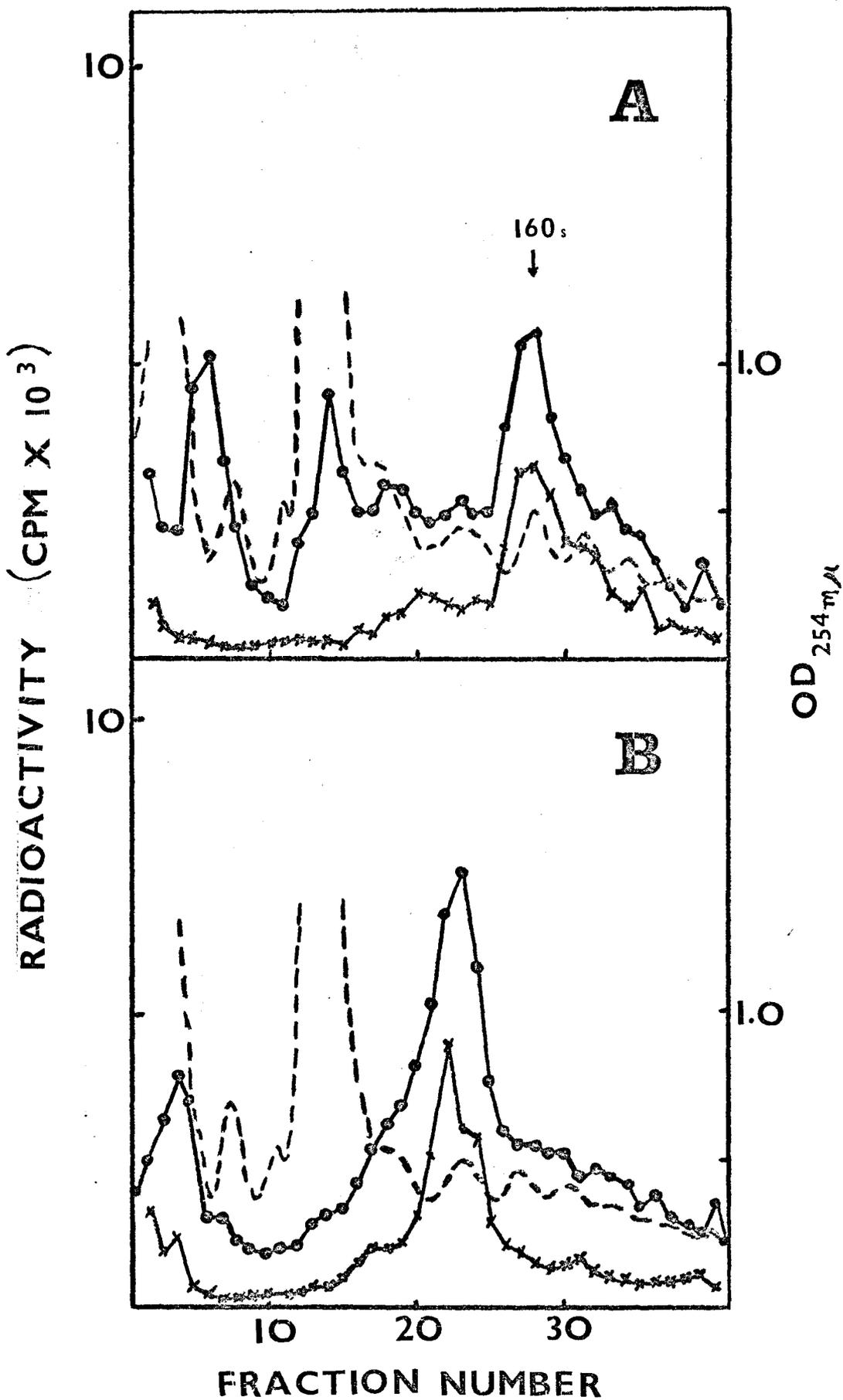
Since Huang and Wagner (1966a) have demonstrated that the T particle is directly responsible for the interference phenomenon, infection of cells in the absence of T particles should minimize interference. A virus stock containing pure B particles (called the PP-2 stock) was obtained by a low multiplicity passage of virus obtained by plaque purification from the HR-LT strain. The absence of a significant number of T particles in this stock was ascertained directly by electron microscopic observation and indirectly by the finding that the titer of the virus was ten times as high as that of the normal HR-LT stock.

3. Cytoplasmic Extract of PP-2 Infected Cells Labelled with ^3H -Uridine

The distribution of the cytoplasmic extract of cells infected with pure B particles and labelled with ^3H -uridine, on a sucrose gradient is illustrated in fig. 16a. As all conditions except the infecting virus were the same as those outlined in section II-1, any difference from the corresponding result in optical density and radioactivity profiles of the gradient is due only to the conditions of low interference. The high absorbance (254 m μ) and ^3H -uridine incorporation in the polyribosome region indicates that there is a very high level of m-RNA present in these cells. Although no unusually high radioactivity (compared to fig. 6b) could be seen present in the 100 and 140S fractions, a substantial peak is present in a fraction cosedimenting with polyribosome tetramers.

fig. 16 Cytoplasmic extract of PP-2 infected cells labelled with ^3H -uridine.

Two cultures of 10^8 cells were infected with the PP-2 preparation of HR-LT VSV at an M.O.I. of 50, treated with Act. D and labelled with ^3H -uridine. The cytoplasmic extract of one culture (A) was layered directly on a sucrose gradient while the other (B) was first treated with DOC (0.5%) then layered on the gradient. The gradients were sedimented, monitored for optical density and checked for RNase sensitivity as outlined in fig. 6c. The optical density (-----), and RNase sensitive (—●—●—) and RNase resistant (—x—x—) radioactivity are plotted.



Prior to being analysed for radioactivity each of the above fractions was split in half and one portion treated with RNase (5 $\mu\text{gm/ml.}$) for 20 minutes at 37°C. Both portions were then precipitated with TCA (5% W/V) and monitored for radioactivity. The results in fig. 16a show that most of the RNA in the gradient is sensitive to the enzyme. However, material sedimenting beyond 120S in the gradient is to some degree RNase resistant with the highest concentration of this resistant material co-sedimenting with the tetramers which have been previously shown to correspond to the 160S region.

If these RNase resistant fractions are assumed to contain RNP as was the case in the previous section, then cells infected under conditions of minimal interference contain RNP which sediments faster than the corresponding RNP in cells infected under high interference.

4. Effects of Sodium Deoxycholate on the Cytoplasmic Extract of PP-2 Infected Cells

The increase in the sedimentation coefficient of the proposed RNP described above could be due to its association with some intracellular structure. Accordingly the above experiment was repeated with the added step that the cytoplasmic extract was treated with 0.5% sodium deoxycholate (DOC) prior to centrifugation on a sucrose gradient. The radioactivity profile of this preparation shown in fig. 16b indicates that the 160S radioactivity peak present in fig. 16a now sediments as 140S. The corresponding peak of RNase resistant material also sediments at 140S.

These results indicate that newly synthesized RNA which is normally associated with a 160S structure in the cells infected under conditions of

low interference, may contain 140S RNP characterized in the previous section, which is associated with some DOC sensitive entity. It was also found that small though significant amounts of RNase resistant material were present throughout the polyribosome region of the above gradients. These could represent breakdown products of the 160S entity or some intermediates in the synthesis of the virus.

5. RNA Present in the 160S and DOC Produced 140S Cytoplasmic Fractions

If the 160S and DOC derived 140S cytoplasmic fractions of the above infected cells contained 43S RNA, the hypothesis that they contain RNP would be confirmed. Accordingly, RNA was extracted from 160S and 140S DOC derived fractions of the infected cells and analysed on a sucrose gradient.

From fig. 17 it may be seen that both cytoplasmic fractions examined contain 43S RNA. This is a further indication that DOC converts the material in the 160S cytoplasmic fraction into material sedimenting at 140S. Since the RNA in these two fractions has the same sedimentation coefficient as B particle RNA, this further supports the hypothesis that these fractions contain RNP.

6. Cytoplasmic Extract of PP-2 Infected Cells Labelled with ¹⁴C-Amino Acids

To further demonstrate that the 160S cytoplasmic fraction contains RNP, PP-2 infected cells were treated with Act. D and labelled with ¹⁴C-amino acids. The cytoplasmic extract of this culture was split into two aliquots. One portion was treated with DOC as in the above experiments, and both were analysed on sucrose gradients. The distribution of

fig. 17 RNA extracted from 160S and DOC-derived 140S cytoplasmic fractions of the PP-2 infected cells.

³H-RNA was extracted with SDS from the 160S and 140S DOC-derived cytoplasmic fractions of PP-2 infected cells prepared as shown in fig. 16. Following precipitation of the RNA with ethanol, it was redissolved in STE-SDS buffer, mixed with ¹⁴C labelled cellular RNA which served as a marker and analysed on a sucrose gradient. ¹⁴C radioactivity (---) and ³H radioactivity (—●—●—●—) are plotted.

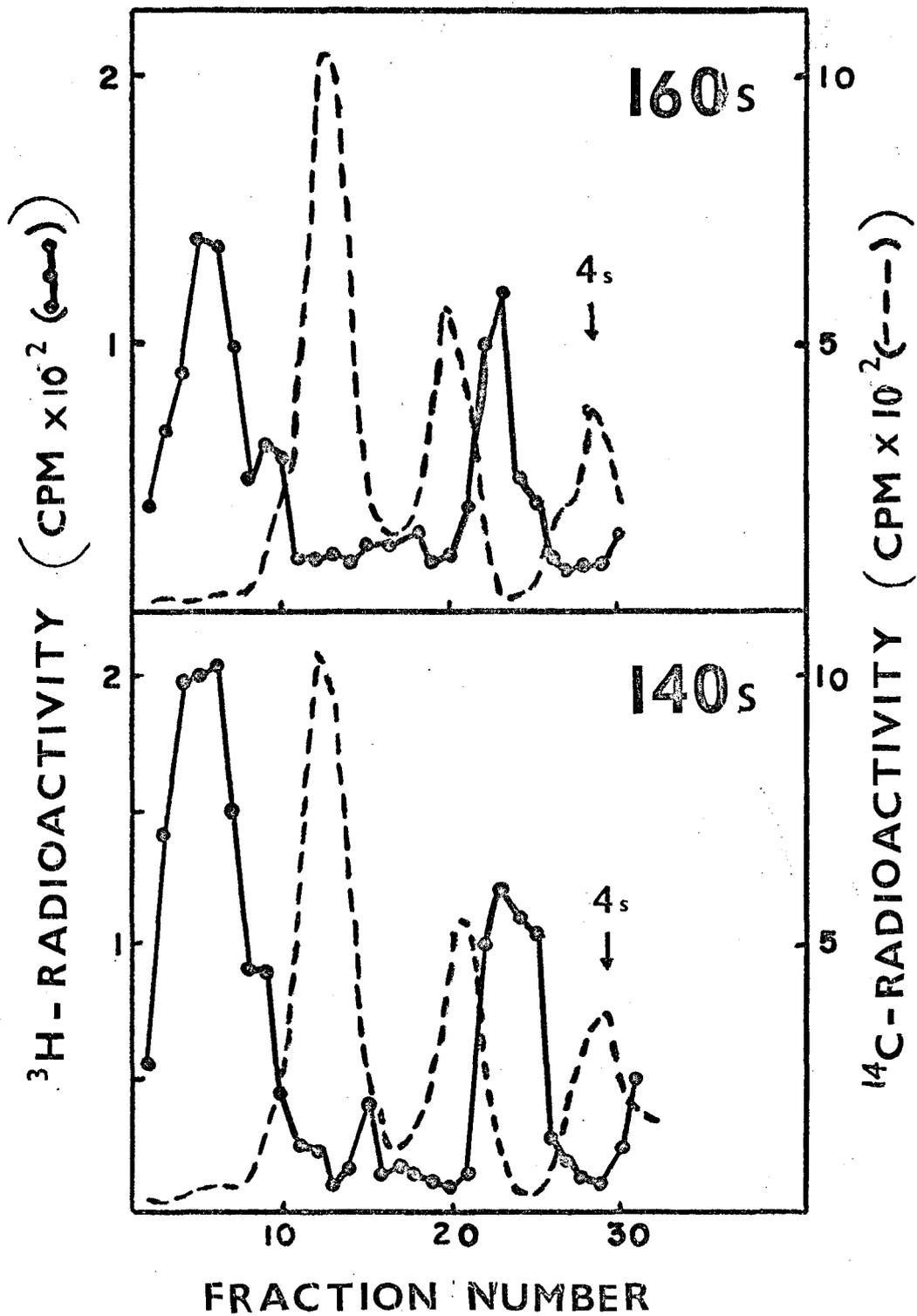
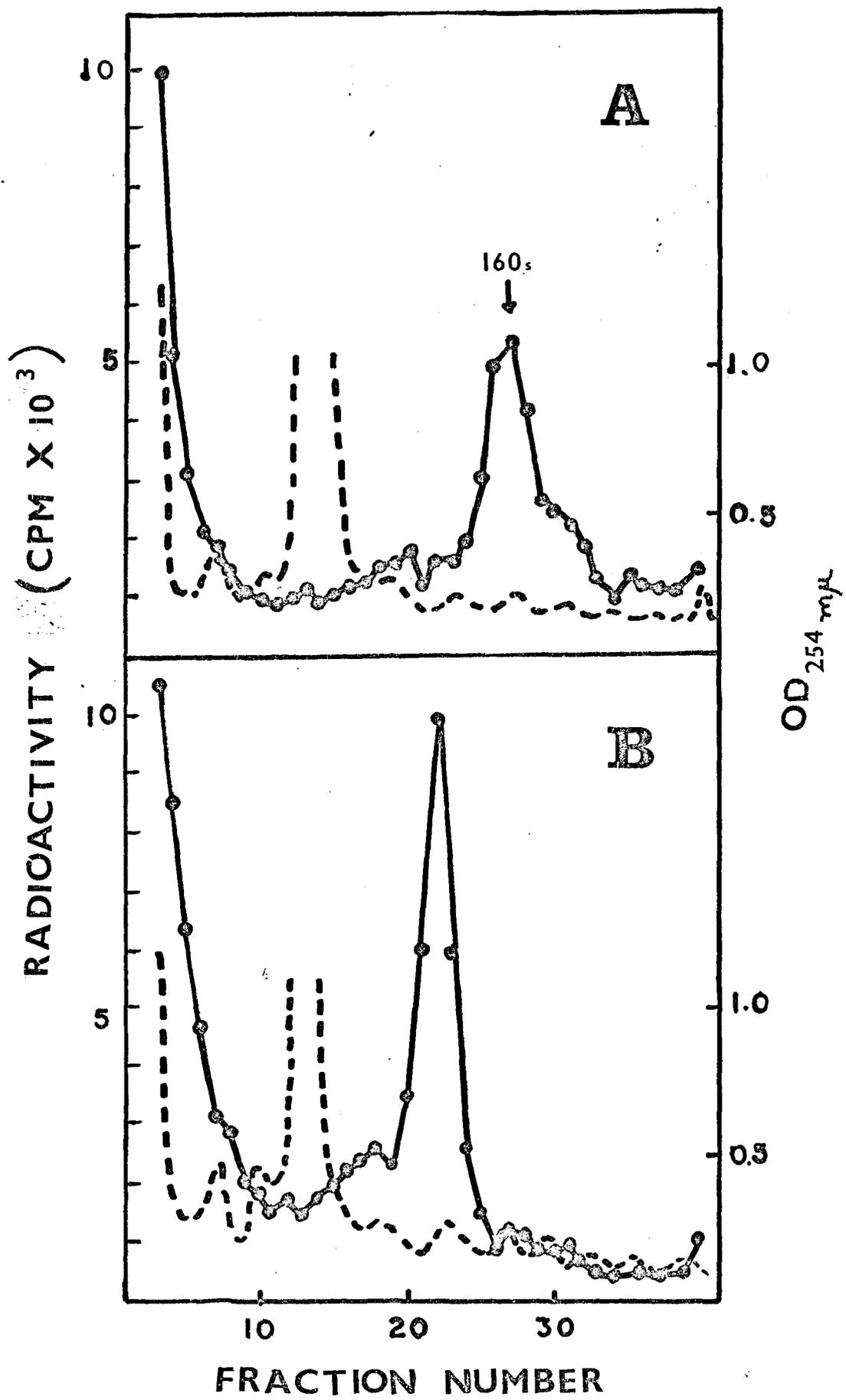


fig. 18 C^{14} -amino acid labelled cytoplasmic extract of PP-2 infected cells.

About 2×10^8 cells were infected at an M.O.I. of 50 with the PP-2 stock of HR-LT strain of VSV. The infected cells were treated with Act. D and labelled with ^{14}C -amino acids in amino acid deficient medium as outlined in fig. 9. The cells were harvested at 5 hours post-infection, cytoplasmic extract was prepared and divided into two equal portions. DOC (to a final concentration of 0.5%) was added to one portion, then both portions were layered on sucrose gradients and analyzed as described in Chapter 2. Optical density (-----) and radioactivity (—●—●—) are plotted.



radioactivity in fig. 18a demonstrates that the newly synthesized protein is concentrated in the 160S fraction. After DOC treatment (fig. 18b) the majority of the newly synthesized protein sediments in the 140S cytoplasmic fraction. A small amount of ^{14}C label is spread throughout the polyribosome region of the gradient, indicating a level of protein synthesis higher than that present in fig. 9 which is the higher interference counterpart of this experiment. The results of this experiment support the hypothesis that the RNP synthesized in virus infected cells under conditions of low interference sediments as a 160S entity which consists of 140S RNP along with a DOC sensitive component.

7. Electron Microscopic Study

Final evidence that the RNP is present in the 160S fraction of cells infected under conditions of low interference comes from an electron microscopic study. Photomicrographs of uranyl acetate stained samples of the 160S fraction contained evidence of RNP complexed with material which resembled membrane. However, the 140S fraction which in this gradient contained only traces of RNP, was found to contain large amounts of RNP identical to that shown in fig. 13b, after DOC treatment. Traces of RNP were found in all other fractions of the polyribosome profile, though this was not unexpected since RNase resistant RNA was found here also.

In summary: cells infected under conditions not favouring interference have within their cytoplasm an RNP complex, which sediments at 160S. This structure appears to consist of a 140S RNP equivalent to that found in the virion which is associated with some DOC sensitive entity. This complex is not found in a significant amount in cells infected under conditions favouring interference.

The following information concerning the replication of VSV in L-cells has been obtained from the results of the work described in this thesis.

1. Infection of L-cells with the wild type (IND-ST) strain of Indiana VSV produces infectious B particles and at least two defective T particles. The shorter defective particle (ST) is produced in larger amounts than the longer defective particle.
Infection of L-cells with the heat resistant (HR-LT) strain of VSV results in the production of infectious B particles and only one type of defective (LT) particle.
2. Virus-specific RNA species with sedimentation coefficients of 15S and 30S are found associated with the polyribosomes of virus-infected cells. These single stranded RNA species may function as messengers in the synthesis of viral proteins.
3. While virus infected cells contain all four viral structural proteins in a ratio similar to that found in the mature virus, only VP-3 is found to any extent in the infected cell cytoplasmic extract.
4. When infection of L cells is carried out under conditions of interference (i.e. both B and LT particles present in the inoculum) there is an accumulation within the cell of ribonucleoproteins with sedimentation coefficients of 100S and 140S which are characteristic of the ribonucleoprotein of LT and B virions respectively. In contrast, cells

which were infected with B particles alone (minimal interference) contain a ribonucleoprotein complex which sediments at 150 to 170S, but which on treatment with DOC can be converted to a structure with a sedimentation coefficient of 140S.

New Defective Particles. The discovery that the HR-LT strain produces a defective particle larger than the major defective particle produced by the IND-ST strain of VSV may be an important step in the investigation of homologous interference and defective virus production. Recent work by Prevec and Kang (1970) has shown that while both LT and ST particles are capable of homotypic interference with the infectious Indiana virus, only the LT particle can interfere with the heterotypic New Jersey VSV infectious particle production. As seen from the work in this thesis the molecular weight of the LT particle genome is probably 1.7 to 2.0×10^6 daltons of RNA, while the genome of the ST particle contains 1.1×10^6 daltons of RNA. As pointed out by Prevec and Kang (1970) the size of the genome of the ST particle is just sufficient to carry the information for the viral proteins VP-2 and VP-4 while the LT genome can code for the viral proteins VP-2, VP-4 as well as VP-3. An investigation of the virus-specific RNA and protein synthesis which occurs during infection with the LT and ST particles may well provide a clue to the biochemical mechanism of interference.

Of equal importance is the observation that heat treatment of the IND-ST strain of VSV leads to the production of a strain having only LT defective particles. Since the IND-ST strain does produce a small amount of LT like particles, a fact which is apparent from the observations of Huang

and Wagner (1966a) and Hackett (1964), it is possible that the high temperature causes a mutation or selects for those particles in the IND-ST population which produce LT. A better understanding of the mechanism involved in this phenomenon should provide fundamental information regarding the basis of defective particle formation.

Viral m-RNA in Infected Cells. In cells infected with an RNA virus functional virus specific m-RNA can be localized in the polyribosomes (Penman et al. 1963). In order to investigate the m-RNA in VSV infected cells the polyribosomes of these cells were examined. The present result shows that an RNA species with a sedimentation coefficient of 15S is associated with these polyribosomes. As the 15S RNA is not found in the virions of VSV, and since it is present in the polyribosomes of infected cells, it appears a likely candidate for viral m-RNA. This messenger function for 15S RNA also proposed by Schaffer et al. (1968) will now be further examined.

A high resolution analysis of 15S RNA has shown that it consists of three distinct species sedimenting at 13, 15 and 18S on a sucrose gradient. The molecular weights of these species calculated from the above sedimentation coefficients proved to be in close agreement with the theoretical molecular weights of monocistronic m-RNA required to code for viral proteins VP-4, VP-3 and VP-2 respectively. The 15S RNA therefore appears to consist of three species of RNA which from theoretical and experimental evidence could serve as m-RNA. In contrast to the poliovirus infected cell systems where the m-RNA is polycistronic (Summers and Maizel 1968), the m-RNA in VSV appears to be monocistronic with each viral protein

being coded for by an individual messenger.

A species of RNA sedimenting at 30S which probably corresponds to the 28S RNA reported by Stampfer et al. (1969), was also found associated with the polyribosomes of the infected cells and hence could have an m-RNA function. Baltimore (personal communication) has also found 28S RNA with the polyribosomes of the cells infected with the wild type Indiana VSV, a strain known to produce very few if any detectable "long T" particles.

It is as yet unknown if the 30S RNA has a base sequence identical to the 15S m-RNA species or whether the 30S RNA represents the remaining portion of the viral genome. From a purely theoretical point of view, either case is possible since the molecular weight of the 30S RNA (1.7×10^6 daltons) could represent the sum of the molecular weights of the other three 15S RNAs or could be related to the remaining half of the viral genome. It is as yet an unresolved matter whether the 15S RNA is copied as such from the 43S viral RNA or if it is copied as a 30S RNA which is subsequently cleaved into the 15S species.

If the 30S RNA does not contain the same base sequence as those present in the 15S species of RNA and is an m-RNA, then there should be a corresponding protein synthesized from this template detected in the infected cells. A likely candidate for such a protein is the yet obscure VP-1 whose molecular weight of about 200,000 daltons could be coded for by a messenger RNA of 1.7 to 2.0×10^6 daltons. This hypothesis accounts for all the genetic information present in the genome of the infectious virus in terms of the structural proteins produced by the virion.

Indirect evidence that 15S and 30S RNA serve as viral m-RNA in infected cells comes from examining the unusual polyribosome profile of infected cells illustrated in fig. 6b. Here the dimer, trimer, and tetramer peaks appear to be partially resolved into a faster and a slower sedimenting component. The above result becomes significant when the difference in the molecular weight of the ribosomes containing the above two species of RNAs are considered. The dimer aggregate containing 30S RNA (9.7×10^6 daltons) would have a 12% greater molecular weight than the corresponding dimer containing 15S RNA (8.6×10^6 daltons). Similarly the molecular weights of two such trimers would differ by 9% and this difference in the molecular weight would become continuously smaller with the larger aggregates of ribosomes. Since the sedimentation coefficient is a function of the molecular weight, then the small ribosomal aggregates, differing only in the molecular weight of their m-RNA may have sufficiently different sedimentation coefficients to sediment as distinct bands in a sucrose gradient. Such a phenomenon is indeed present in fig. 6b discussed above.

These studies have shown that there may be four monocistronic messengers present in the VSV infected cells. A study of the temporal production of these species of RNA may be instrumental in the understanding of the replication phenomenon of VSV.

Viral Protein and RNP in the Infected Cell. From electron microscopic studies of the virus infected cells, Howatson and Whitmore (1962) postulated that the VSV virion is assembled as it buds through the cell membrane. An

examination of the proteins present in the virus infected cells showed that all the viral structural proteins were present in a ratio not unlike that found in the virus. However, the cytoplasmic extract of the infected cells prepared as described in Chapter 2 contained only VP-3 to any significant degree. It therefore appears that the coat proteins VP-2 and VP-4 are associated with structures which are removed with nuclei during the cell fractionation. It is likely that these structures are on the cytoplasmic membranes. On the other hand, VP-3, the RNP protein, remains in the cell as RNP and as soluble protein (fig. 10). While VP-4 appears to form a complex with the cell membrane (Wagner et al. 1969b), the VP-2 may be in part membrane associated and in part released into the extracellular fluid as a soluble 6S antigen (Kang and Prevec 1969).

The mechanism of the association of RNP with the membrane-associated viral coat proteins to form mature virus remains as an important problem for further investigation. In this connection it is of interest that cells infected with pure B particles show 140S RNP associated with a DOC sensitive component in a 160S structure. It is not inconceivable that this represents a stage in the development of mature infectious virus. The absence of the 160S structure in cells infected with a mixture of B and LT particles and the presence instead of free 100S and 140S RNP in the cells may be related to the process of interference which is occurring in this infection. More work will have to be done to determine the functional significance of this result.

In this regard it is of interest that Baltimore et al. (1970) have

shown that disrupted infectious VSV particles sedimenting between 100 to 200S contain functional RNA specific RNA polymerase. Evidence was shown by Galet (personal communication) that the polymerase activity in cells infected with B particles alone is much higher than in cells infected with B and T particles. Hence there could be a correlation between the presence of the 160S RNP complex and the polymerase activity in the cell.

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