RNA COMPLEXES IN VESICULAR STOMATITIS VIRUS INFECTED L-CELLS

STUDIES ON THE RNA AND RNA COMPLEXES PRODUCED BY VESICULAR STOMATITIS VIRUS IN MOUSE L-CELLS

by

DOUGLAS J. HALLETT, B.Sc.

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AUTHOR: Douglas J. Hallett, B.Sc. (McMaster)

SUPERVISOR: Dr. L. A. Prevec, Ph.D.

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Virus specific RNA components of the cytoplasmic extracts of cells infected with the Indiana serotype of vesicular stomatitis virus were examined. Studies were carried out both in the presence and absence of defective particle interference.

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TABLE OF CONTENTS

Page

INTRODUCTION 1

Purpose of the Study 12

MATERIALS AND METHODS 14

1. Source of Virus 14
2. Growth of Medium 14
3. Chemicals and Solutions 15
4. Biological Compounds and Radiochemicals 16
5. Source and Growth of Cells 17
6. Assay for Plaque-Forming Units (PFU) 17
7. Plaque Purification 18
8. Infection with Virus 18
9. Preparation of Virus Stocks 19
10. Labelling of Cells with Radioisotopes 19
11. Extraction of Cytoplasm from Infected Cells 20
12. Preparation and Analysis of Sucrose Gradients . . . 21
13. Purification of Virus 23
14. Extraction of RNA 24
15. Hybridization of Messenger RNA's to Virion RNA . . 26
16. Electron Microscopy 26

RESULTS 28

I. Viral RNA Species in the Cytoplasm of Cells During Infectious Virus Production 28

TABLE OF CONTENTS (continued)

Page

RESULTS (continued)

A. Characterization of RNA from the Gradient Fractions Coincident with the 30S and 50S Ribosomal Subunits 31

B. Hybridization of 27S and 15S Messenger-RNA Species with LT Particle Virion RNA . . . 36

C. Characterization of the RNA from the Heavy Complex Coincident with the Subviral Ribonucleoprotein Marker 38

D. Demonstration that the Heavy Complex is a Ribonucleoprotein Complex 42

II. RNA Sequences in the Cytoplasm of Cells Infected Under Conditions of Defective Particle Production 46

A. ST Particle Interference 50

B. LT Particle Interference 53

DISCUSSION 58

A. Viral Specific Messenger RNA 58

B. Free Negative 27S RNA Found in Cells Producing Infectious B Particles 60

C. The RNP Complex Formed Under Conditions of Infectious B Particle Production. 61

D. RNP Complexes Found Under Conditions of LT and ST Defective Particle Interference 63

BIBLIOGRAPHY 68

LIST OF FIGURES

Figure	Title	Page
1	Cytoplasmic Extracts of Cells During Infectious Virus Production	29
2	Messenger-like Viral RNA Species Coincident with the 40S and 60S Regions	32
3	Hybridization of mRNA to Infective B Particle Virion RNA	34
4	Hybridization of 28S and 15S Messenger RNA Species of LT Particle Virion RNA	37
5	Comparison of the RNA Extracted from the RNP Region and Infectious Virions	39
6	Hybridization of RNA Extracted from Subviral RNP Complex to RNA of Infective B Particle Virions	41
7	Resistance of a Cytoplasmic Extract to Digestion with Pancreatic Ribonuclease	43
8	Electron Micrograph of Material Taken from Fractions Containing the RNP Complex Found in EDTA Gradients	45
9	Sedimentation Characteristics of B, ST and LT Particle Virions on Linear Sucrose Gradients	47
10	RNA Extracted from LT and ST Virions	48
11	Cytoplasmic Extract of Cells Infected Under ST Particle Interference Conditions	49
12	RNA from RNP Complexes Found in Cells Infected Under ST Particle Interference Conditions	51
13	Cytoplasmic Extract of Cells Infected Under Conditions of LT Particle Interference	54
14	RNA Extracted From the RNP of Cells Infected Under Conditions of LT Interference	56

INTRODUCTION

A virus is an extremely simple organism when compared to the living cell. It consists of a nucleic acid genome, either RNA or DNA, enclosed in a specific protein or protein-lipid shell. Viruses lack the enzymes necessary for energy metabolism and have an obligatory dependance on a host cell for their replication. The relatively simpler molecular events of virus replication are being explored to test ideas and provide models for the more complex regulation of cellular growth and division. An understanding of virus replication, *per se*, is of both medical and economic importance as viruses produce disease in most forms of living organisms.

The mode of expression of animal virus genomes has been recently reviewed by Baltimore (1971). The essential features of virus replication are the synthesis of virus specific messenger RNA (transcription), this messenger RNA then being used to specify the synthesis of virus specific proteins (translation), and the synthesis of new nucleic acid genomes of the virus (replication), these new genomes combining with virus protein to form new virus particles. The replication of most RNA viruses in suitable hosts can proceed in the absence of both DNA synthesis and DNA directed RNA synthesis. Because of this latter fact, the antibiotic Actinomycin D (Act D) can be used to suppress host cell RNA synthesis and thus allow examination by isotope incorporation of virus-specific RNA synthesis free from cellular RNA synthesis. The exceptions to the above statement are the myxovirus (influenza) group which have an early Act D sensitive step in replication and the RNA tumour viruses which require both DNA synthesis and DNA dependent RNA synthesis for replication (Spiegelman _et_ _al_., 1970).

Following the convention proposed by Baltimore, i.e., that molecules with messenger function be termed positive and their complementary strand be termed negative, viruses can contain single-stranded RNA genomes which are, at least in part, positive, such as the picorna and arboviruses, or negative, such as the rhabdo, myxo, and paramyxo virus groups. In this latter case a virion-associated transcriptase enzyme must be carried into the host cell with the virus genome in order to initiate infection (Baltimore _et_ _al_., 1970; Huang _et_ _al_., 1971). Virion-associated transcriptase molecules have also been found associated with the double-stranded DNA vaccinia virus which replicates in the cell cytoplasm (Kates _et_ _al_., 1967), and the double-stranded RNA reovirus (Borsa and Graham, 1968).

The genomes of RNA viruses may be segmented as in the case of reovirus, each segment possibly coding for a single monocistronic messenger RNA. Picornavirus have a positive stranded RNA genome which acts as a polycistronic template for synthesis of a large single protein molecule which is then cleaved by proteolytic enzymes to form functional virus proteins (Jacobson and Baltimore, 1968). This messenger RNA therefore has a single initiation and termination site. Vesicular stomatitis virus (VSV), a rhabdovirus with a negative stranded genome, makes RNA species which can be isolated from infected cell polyribosomes. These RNA species are complementary to the virus genome (Huang et al., 1970) and correspond in size to the size of genome required to code for each individual VSV protein (Petric and Prevec, 1970; Mudd and Summers, 1970). It was postulated that the different species of virus specific messenger each code for one viral protein.

The general history and morphology of VSV has been reviewed by Howatson (1970). It infects a broad range of animal hosts (Hanson, 1952) and may be transmitted by arthropod vectors such as mosquitoes. The symptoms of VSV in cattle resemble those of foot and mouth disease except that the disease is seldom fatal and recovery is usually rapid and complete. A strain of the Indiana serotype was used in the experiments of this thesis. However different strains such as the New Jersey strain (Myers and Hanson, 1962) and Cocal strain (Jonkers et al., 1964) have been shown to have some antigens in common with the Indiana strain.

The morphology of the virus has been examined by electron microscopy of thin sections of infected cells and negatively stained viral preparations (Howatson and Whitmore, 1962; Nakai and Howatson, 1968). Infectious particles are bullet-shaped and are therefore designated B particles. They consist of an internal helical structure surrounded by a membranous envelope with fine outer filamentous projections. B particles were measured to be 175 nm in length and 65 nm in diameter, with surface projections some 10 nm long. Nakai and Howatson suggest that the nucleoprotein core forms a single helix of about thirty turns capped by four or five turns of diminishing diameter at the round end. The continuity of the helix is maintained by a single-stranded covalently bonded RNA molecule. All VSV particles contain four major structural proteins designated L for large, G for glycoprotein, N for nucleoprotein, M for matrix and one minor protein designated NS (Kang and Prevec, 1969, 1970, and 1971). Proteins G and M are components of the viral envelope (Kang and Prevec, 1969; Wagner et al., 1969) whereas protein N is a component of the internal nucleoprotein. Proteins L and NS are associated with the internal nucleoprotein but are readily dissociated with high salt (Emerson and Wagner, 1972).

When VSV is repeatedly passed undiluted at high multiplicity in cell culture the yield of infectious virus is reduced and shorter truncated defective particles appear as viral products (Cooper and Bellet, 1959). Von Magnus (1954) observed a reduction in the number of infective particles produced after several serial passages of influenza virus. Defective virus have been shown to be responsible for viral interference resulting in a Von Magnus type phenomenon in several virus systems (Huang and Baltimore, 1970). Defective particles are deficient in some aspect necessary for their own replication and are not infectious. They have been found in many different mammalian virus systems (Uchida and Watanabe, 1969; Parks et al., 1968; Pons and Hirst, 1969; Huang and Baltimore, 1970; Mak, 1971; Cole et al., 1971). The defective particles of VSV were shown to be responsible for the interference phenomenon when detectable interferon synthesis was not induced (Huang and Baltimore, 1970).

The Indiana strain of VSV produces two types of defective particles. The original parental strain of Indiana VSV produces predominately a particle 65 nm in length and diameter (Huang et al., 1966). Petric and Prevec (1970) observed that a heat-resistant strain of Indiana VSV (IND-HR) produced defectives that were 100 nm in length and 65 nm in diameter. Both LT and ST particles contain helically-wound ribonucleoprotein (RNP) structures morphologically similar to the RNP of infectious B particles but shorter in length (Nakai and Howatson, 1968; Petric and Prevec, 1970). No difference in the structural proteins of the B particles and defective T particles could be detected (Kang and Prevec, 1969; Wagner *et al.*, 1969). Defective particle base sequences are not complementary to B particle RNA or to each other (Bishop and Roy, 1971).

VSV multiplies rapidly and produces a cytopathic effect in many different cell cultures. The defective particles produced are easily separated from each other and from infectious particles by rate zonal centrifugation on sucrose gradients and are readily identified by sedimentation coefficient and morphological characteristics. These features make VSV an ideal model system for the study of both biological and biochemical aspects of defective particle production and virus interference.

Much information has been accumulated on the homotypic interference phenomenon of VSV: (a) Simultaneous infection of cells by B and T particles lowers the yield of infectious virus produced (Cooper and Bellet, 1959); (b) interference does not occur at the level of virus adsorption or penetration since addition of defective particles to infected cells anytime within the virus latent period (1.5 hours) can reduce virus yield (Huang and Wagner, 1966); (c) addition of defective particles to infected cells after the latent period has no effect on virus yield showing an early virus function is inhibited (Huang and Wagner, 1966); (d) U.V. irradiation of defective particles abolishes their interfering activity, suggesting that intact defective virion RNA is required for interference (Huang *et al.*, 1966); (e) RNA isolated from ST particles causes interference with B particle production in chick embryo culture (Sreevalson, 1970); (f) T particles are not infectious and replicate only in the presence of B particles (Huang and Wagner, 1966).

As mentioned previously VSV carries a virion-associated transcriptase enzyme. RNA specific RNA transcriptase activity has also been obtained from infected cell extracts (Wilson and Bader, 1965; Galet, Shedlarski and Prevec, in press). Over 90% of the product transcribed by this virion-associated enzyme from B particles is complementary to the virion RNA. Some transcriptase activity is found associated with LT particles but none with ST particles (Bishop and Roy, 1971). This *in vitro* transcriptase work has provided possible mechanisms of virus specific messenger RNA synthesis. No virus-specific replicase enzyme has yet been isolated in VSV infected cells. Attempts have been made to attack this problem by looking at the viral RNA products found in the cytoplasm of VSV-infected cells. Previous work of this nature has been done with virus stocks containing defective particles. Some species of RNA found in cells infected with these stocks were therefore replicative forms and replicative intermediates of the defective particles making the identification of replicating forms of infective particles very complex.

Different researchers have found the RNA of defective virions to have different sedimentation coefficients. Schincariol and Howatson (1970) found the virion RNA of infectious B particles of the IND-HR strain to sediment at 38S and the defective LT particles to sediment at 26S. This is in contrast to Petric and Prevec (1970) who found the LT particle virion RNA to sediment at 30S. The ST defective particle produced by the original IND strain was shown by Huang and Wagner (1966) to contain RNA having a sedimentation coefficient of 23S while the B particle produced by this stock contained RNA with a sedimentation coefficient of 43S. Schincariol (1971) showed the RNA of his ST particle to sediment at 21S. Whether these differences are a property of this viral RNA or of the experimental method is not clear.

Bearing these differences in mind, Schincariol and Howatson (1970) showed single-stranded species of RNA with sedimentation coefficients of 13, 15, 21, 26, 31 and 38S in the cytoplasm of cells infected with the IND-HR stock. The particle yield of this infection consisted of 85% infectious particles and 15% defective LT particles. The 26S and 38S components have the same sedimentation coefficients as the RNA of LT and B particles respectively. Two classes of double-stranded RNA sedimenting at 13 to 15S, and 19 to 20S are found in small quantities and it was postulated that these are replicative forms of LT and B particles respectively. Partially ribonuclease resistant RNA species sedimenting heterogeneously from 13S to 50S were also found and it was postulated that these represent replicative intermediate complexes.

Using an IND-HR-LT strain of VSV which produced 50% defective LT particles and 50% infective B particles, Petric and Prevec (1970) showed the presence of 100S and 140S ribonucleoprotein complexes in Mg^{++}-containing sucrose gradients of the cytoplasmic extracts of the infected cells. Under these conditions of isolation the structure cosedimented with polyribosomes carrying VSV specific messenger RNA. The 100S RNP contained RNA sedimenting at 30S which corresponded to the sedimentation coefficient of LT particle RNA. The 140S RNP contained RNA sedimenting at 43S corresponding to the sedimentation coefficient of B particle RNA. These complexes were designated subviral as they appeared equivalent to the nucleoprotein structures released by detergent treatment of LT and B particles. RNA sedimenting at 30S and 15S apparently associated with the polyribosomes was also found in the RNP-containing fractions of the gradients.

Recently, Kiley and Wagner (1972) have isolated three intracellular viral ribonucleocapsids with sedimentation coefficients of 140S, 110S and 80S from infected cells producing B, LT and ST virions corresponding respectively to the above RNP complexes. All three nucleocapsids contained three viral proteins L, N and NS. The RNA extracted from the three nucleocapsid species sedimented at 42S, 28S and 23S which roughly corresponded to the RNA extracted from B, LT and ST virions respectively.

Huang *et al*. (1970) dissociated the polyribosomes actively translating VSV specific RNA in infected cells by treating them with the chelating agent EDTA. EDTA binds divalent cations such as Mg^{++} ions which are necessary for ribosomal integrity. After EDTA treatment species of RNA were found which sedimented at 28S and 15S. These species were single-stranded and hybridized completely to B particle virion RNA. Since these species were originally present in the polyribosome region, and moved from this region when the polyribosomes were dissociated with EDTA, they are thought to be virus-specific messenger species. Later Petric and Prevec (1970) and Mudd and Summers (1970) separated the 15S size class of messenger RNA species showing it to be a more heterogeneous species of a least three size classes. If these messengers are monocistronic and assuming one dalton of polypeptide is equivalent to 10 daltons of RNA these mRNA species were matched to three viral-specific proteins.

More recently Huang and Manders (1972) have shown that in the presence of cycloheximide (an antibiotic which specifically blocks protein synthesis) added at the time of infection, input virion RNA is completely transcribed into viral-specific RNA species with sedimentation coefficients of 28S and 13 to 15S. Sequences of polyadenylic acid (Poly A) which represent about 25% of the total ^{3}H-adenosine incorporated into the RNA were found in the 13 to 15S messenger species but not in virion RNA. Poly A sequences were also found in the 28S messenger species (Huang, personal communication).

Models of VSV defective particle interference have been proposed (Prevec and Kang, 1970). The idea basic to most models is that the defective particles because of their shorter genome do not carry all of the genetic information necessary for infectivity although they do contain all of the virus-specific proteins found in the infective particle. It seems logical that the defective particles are deletion mutants for a number of cistrons coded by the infectious genome. Since no complementarity has been demonstrated between defective particles and temperature-sensitive mutants of the virus, it remains to be determined whether the genomes of the defective particles can be transcribed in the cell (Reichmann *et al.*, 1971).

There are several ways that the defective particles can interfere with infective particle production. Huang and Manders (1972) have shown that addition of defective T particles at either one hour before or at the same time as infective B particles to cycloheximide treated cells does not alter the rate of transcription nor does it change the sucrose gradient distribution of viral RNA species produced in infected cells. They propose, therefore, that defective T particles interfere at the stage of replication of viral RNA synthesis.

Purpose of the Study

Previous work on the RNA species found in VSV-infected cells has been done using stocks which produced defective particles as well as infective B particles. As a result many different RNA species were observed. In order to simplify and reduce the number of RNA species a stock was prepared that produced only infective B particles. Studies were carried out on the RNA species found in the cytoplasm of cells infected with this stock.

RNP complexes have been shown to cosediment with polyribosomes on sucrose gradients of cytoplasmic extracts of infected cells. These polyribosomes could be dissociated on sucrose gradients containing EDTA buffer leaving RNP complexes free of polyribosome-associated messenger RNA. RNP complexes were therefore obtained by centrifugation of cytoplasmic extracts of cells infected under conditions of B particle, LT particle, and ST particle production on sucrose gradients in EDTA buffer. The RNA of these complexes was analyzed.

The complementarity of messenger RNA species with B particle and LT particle virion RNA were also examined.

MATERIALS AND METHODS

MATERIALS

1. Source of Virus

Two strains of the Indiana serotype of VSV were used. One strain designated IND-ST was obtained from Dr. A. Howatson of the University of Toronto and was identical to that previously reported (Nakai and Howatson, 1968). The other strain producing LT particles was originally derived by Martin Petric from the heat-resistant strain IND-HR and was designated HR-LT (Petric and Prevec, 1970).

2. Growth of Medium

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

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

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

3. Chemicals and Solutions

All chemicals used were of analytical reagent grade.

(a) Phosphate-Buffered Saline (PBS). (Dulbecco and Vogt, 1954)

NaCl	8.00 gm
KCl	0.20 gm
$NaHPO_4$	1.15 gm
KH_2PO_4	0.20 gm

made up to one liter with glass distilled water, pH 7.5.

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

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

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

(c) EDTA Buffer

EDTA	0.037 gm
Tris	1.21 gm

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

(d) STE-SDS Buffer. (Prevec, 1965)

NaCl	5.8 gm
EDTA	0.37 gm
Tris	1.21 gm
SDS	5.0 gm

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

4. Biological Compounds and Radiochemicals

Actinomycin D (Act D) was a gift from Dr. W. D. Dorian of Merck, Sharpe and Dohme, Montreal. Pancreatic ribonuclease was purchased from Worthington Biochemical Corporation, Freehold, New Jersey.

Amino acid mixture labelled with ^{14}C was purchased from the New England Nuclear Corporation. The approximate specific activity was 1.54 mc/mgm. ^{3}H-uridine was purchased from Amersham-Searle with a specific activity of 29 Ci/mmole.

Toluene base scintillation fluid was prepared by dissolving 4 gm of Omnifluor (New England Nuclear Corp.) in one liter of toluene.

METHODS

5. Source and Growth of Cells

A continuous passage line of Earle's L-cells (Earle, 1943) designated L-60 was obtained from Dr. A. F. Howatson, Department of Medical Biophysics, University of Toronto. These L-cells were grown in suspension culture in MEM supplemented with 5% NBCS. The suspension cultures were kept in tightly stoppered Erlenmeyer flasks, each containing a sterile teflon coated magnetic bar and incubated at 37°C in a water bath equipped with magnetic stirrers.

The cell concentration was measured using a Levy ultraplane hemocytometer and the culture was kept in exponential growth phase by daily two-fold dilution keeping the concentration between 4 and 7 x 10^5 cells/ml.

6. Assay for Plaque-Forming Units (PFU)

To determine the plaque-forming titer after harvesting virus, 0.1 ml of virus, after several dilutions in PBS, was pipetted directly on a full L-cell monolayer, preformed on Falcon petri dishes (60 x 15 mm). The virus was allowed to adsorb for 30 minutes at 37°C in an air atmosphere containing 5% CO_2 and 100% humidity. The infected cell sheet was then overlaid with 5 ml of MEM growth medium containing 5% NBCS, and 0.9% Noble agar (Difco). Plaques could be counted after incubation for 20 to 24 hours at 37°C in a water saturated atmosphere containing 5% CO_2. The cell sheet was fixed with Carnoy fixative (3 volumes of absolute ethanol to 1 volume of glacial acetic acid) and the agar then washed away revealing the clear plaques.

7. Plaque Purification

Plaque picking for virus purification was carried out to produce a B particle stock. A sterile Pasteur pipette was inserted through the unfixed agar over a visible plaque. The area of the plaque was scraped with the pipette tip removing a small amount of fluid along with an agar plug. This was dispersed in 1 ml of growth medium and was retitered before each subsequent plaque pick.

8. Infection with Virus

Exponentially growing cells at from 4 to 6 x 10^5 cells/ml were centrifuged at 400 x g for 10 minutes. The cell pellet was resuspended to 10^7 cells/ml with MEM containing 2% NBCS and the appropriate virus concentration. The virus was allowed to adsorb by incubating for 30 minutes at 37°C in suspension culture, then MEM containing 2% NBCS was added to produce a final cell concentration of 0.5 x 10^6 to 10^6 cells/ml. The time of dilution was designated the beginning T_o of infection. The culture was further incubated in suspension culture at 37°C for the desired period of time.

9. Preparation of Virus Stocks

B particle stock was obtained by five successive plaque purifications and subsequent passage of the virus at a multiplicity of infection (MOI) of 0.01 PFU per cell. After incubation for 8 to 14 hours the cells and cellular debris were removed by centrifugation at 400 x g for 10 minutes and the supernatant stock virus suspension aliquoted and stored at -60°C or -70°C until further use. Virus titers were the order of 10^9 PFU/ml. The stock contained no detectable defective particles as determined by sucrose gradient centrifugation analysis and electron microscopy.

In order to obtain virus preparations containing a substantial proportion of defective VSV particles the appropriate parental virus type (HR-LT for LT defective and IND-ST for the ST defective particle) were passed repeatedly at MOI's of approximately 100 PFU/cell. After four or five such passages the resultant virus stock contained approximately equivalent numbers of B and T particles as determined by either optical density analysis (254 mμ) of linear sucrose gradients or electron microscopy.

10. Labelling of Cells with Radioisotopes

Infected cultures were treated with Act D (2 μg/ml) at 30 minutes and ^{3}H-uridine (2 μc/ml) at 180 minutes post infection.

Labelling with ^{14}C-amino acids and ^{3}H-uridine differed from the procedure described above in that MEM containing 2% NBCS but only one-twentieth the normal concentration of amino acids was used at all stages of infection. Act D was added to the infected cultures at 30 minutes post infection and ^{14}C-amino acids (0.2 μc/ml) and ^{3}H-uridine (2 μc/ml) at 180 minutes post infection.

11. Extraction of Cytoplasm from Infected Cells

At 6 hours post infection the cells were collected by centrifugation at 1000 x g for 5 minutes at 4°C in an International 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 EDTA buffer and the cells were allowed to swell for two minutes. The cells were then disrupted by 10 strokes of a tight fitting Dounce homogenizer and the homogenate centrifuged at 7000 x g and 4°C in a Sorval RC-2B centrifuge. This yielded a pellet consisting of nuclei and cell membranes, and a cloudy supernatant which was subsequently layered on 15 to 30% sucrose in EDTA buffer linear gradients.

12. Preparation and Analysis of Sucrose Gradients

Linear gradients of 5 to 30% sucrose in PBS, and 15 to 30% sucrose in either RSB° or EDTA buffer were prepared in 37 ml volumes in Beckman nitrocellulose tubes using a Buchler gradient maker. The tubes were placed in Beckman SW27 rotor buckets and chilled to 4°C. A sample volume of 1.0 ml was layered on these gradients with a Pasteur pipette. Centrifugation was carried out for various times at 4°C and 81,000 x g with a Beckman SW27 rotor in a Beckman L2-65B or L3-50 ultracentrifuge. The sucrose gradients were then collected in successive 1 ml fractions with continuous monitoring of the optical density (O.D.) (at 254 mμ) of the effluent using an Isco UV analyser and fraction collector. This instrument collects a gradient beginning at the top of lighter portion.

For radioactive analysis 0.2 ml of each fraction was made 5% (w/v) in ice cold TCA and the resultant precipitate collected with suction on a nitrocellulose filter (0.45 μ Sartorius). The acid precipitate was washed with 5 ml of ice cold 5% TCA using suction filtration and the filters dried in an oven at 100°C.

For the analysis of ^{3}H-uridine labelled RNA, linear gradients of 5 to 20% sucrose in STE-SDS were similarly prepared in 5 ml volumes in nitrocellulose tubes and kept at room temperature. A volume of 0.1 ml of material was layered on the gradient using a 100 λ pipette after placing them in Beckman SW50 rotor buckets. Usually ^{14}C-uridine labelled ribosomal RNA (8000 cpm) was also layered on the gradient to serve as an internal standard for sedimentation. Centrifugation was carried out at 15°C with a Beckman SW50 rotor for either 2 hours at 185,000 x g or 3 hours at 107,000 x g (after the rotor was derated) in the Beckman centrifuges mentioned before.

These 5 ml gradients were collected in drops through a hollow needle from the bottom of the nitrocellulose tube directly onto Sartorius glass fibre filter pads placed in glass scintillation counting vials. In this case each collected fraction was approximately 0.13 mls in volume. The glass fibre filters were dried at 100°C prior to the addition of scintillation fluid.

Similar 5 ml linear sucrose in STE-SDS gradients were used to purify ^{3}H-uridine labelled RNA for use in hybridization experiments. In this case unlabelled ribosomal RNA was used as an internal marker for sedimentation. These gradients were collected in successive 0.25 ml fractions with continuous monitoring of the optical density (254 mμ) of the effluent in order to detect the position of the marker using an Isco UV analyser and fraction collector. Then 0.05 ml of each fraction was taken using a 50 λ pipette and TCA precipitated as described before.

Filters were immersed in 5 ml of toluene based scintillation fluid and radioactivity monitored in Beckman LS250, LS233, LS230 or LS100 liquid scintillation counters.

13. Purification of Virus

Cells were infected with the appropriate stock of virus and labelled with ^{3}H-uridine if radioactive virus was desired, all as described previously. After removing the cells and cellular debris from the medium by centrifugation the virus was harvested from the cell free supernatant by centrifugation at 32,000 x g in a Beckman type 19 rotor for 165 minutes. The viral pellets were resuspended in 1 ml of PBS and layered on 37 ml preformed cold 5 to 30% sucrose PBS gradients. The gradients were run at 81,000 x g for 35 minutes. Under these conditions the virus particles form discrete light-scattering bands which are easily visible if the gradient is illuminated from below. Virus was harvested from the gradient by collecting these bands using a J-shaped Pasteur pipette. The open tip of the pipette was inserted carefully through the sucrose gradient to the base of the visible band and the band was aspirated into the pipette.

In other instances when radioactive virus was being purified the gradient fractions were collected on the Isco fractionator and the optical density monitored as described in section 12. The appropriate fractions were pooled for subsequent procedures.

Infectivity titrations and electron microscopic observation of the collected virus material was routinely carried out at this stage.

The purified virus was then pelleted out of sucrose solution by centrifugation at 81,000 x g for 75 minutes and then resuspended in 1 ml of PBS. LT virions were taken through two successive purifications to ensure greater freedom from contamination with infectious B virions.

14. Extraction of RNA

Fractions of interest from linear sucrose gradients of cytoplasmic extracts of concentrated virus were pooled to a maximum volume of 1.5 mls. This was made up to 3.0 mls in STE-SDS and enough 5% SDS solution was added to give a final concentration of 0.75% SDS. RNA was then extracted from the virus or cytoplasmic fractions by a modification of the method of Penman (1966). Water-saturated phenol pre-heated to 60°C and chloroform containing 1% isoamyl alcohol were added in separate 3 ml portions. This mixture was kept in a water bath at 60°C for two minutes and shaken periodically. The tubes were then placed in a cold 4°C Sorval centrifuge and centrifuged at 27,000 x g for 1 minute to separate the phases. The clear upper aqueous layer was removed carefully with a Pasteur pipette and the entire extraction procedure was repeated until no white precipitate was visible at the interface of the aqueous and organic phases. Generally this required three repetitions. Phenol in the final aqueous phase was removed by shaking with two volumes of diethyl-ether and removing the lighter ether phase. This was repeated twice and residual ether then bubbled off with air.

The final aqueous phase was made approximately 0.2 M in NaCl and 2 volumes of 95% ethanol prechilled to -20°C were added. Several drops of yeast RNA dissolved in STE-SDS were added as a co-precipitant, the mixture shaken well, and then left overnight at -20°C. The white flocculent precipitate which formed was collected by centrifugation at 12,000 x g for 15 minutes and re-dissolved in 0.2 ml of STE-SDS. This ethanol precipitation procedure was repeated with material pooled from linear sucrose STE-SDS gradients in section 12 in order to use the RNA for hybridization experiments. The RNA was recollected and dissolved in 0.2 ml of 0.01 M Tris.

In the case of extracted unlabelled viral RNA no co-precipitant was added during ethanol precipitation and the RNA pellet was dissolved in 0.01 M Tris. The concentration of this RNA was determined spectrophotometrically using the relationship that one optical density unit at 260 mμ corresponds to 40 μg of RNA when the ratio of absorbance at 280 mμ to that at 260 mμ is 0.6 (Oda and Jocklick, 1967 from Warburg and Christian, 1942).

15. Hybridization of Messenger RNA's to Virion RNA

Hybridization was used to study the homologous base sequences of the virion RNA of infective B and defective LT particles. Appropriate amounts of virion and messenger RNA were put into 5 ml glass tubes and the volume brought to 0.4 ml with glass distilled water. The tubes were placed in boiling water for two minutes to denature hydrogen bonded regions. The hot solutions were made 0.3 M in NaCl and 0.03 M in Na-citrate by adding 0.1 ml of five times concentrated saline-citrate solution. The tubes were stoppered to prevent evaporation and the RNA was annealed by incubation at 65°C for 2.5 hours. After this time the samples were chilled in ice water and divided into two equal portions. One portion was acid precipitated with a drop of yeast RNA carrier onto nitrocellulose filters for determination of total radioactivity while the other portion was incubated at 37°C for 30 minutes with pancreatic ribonuclease at a concentration of 50 μg/ml prior to acid precipitation and radioactive determination as described in section 12.

16. Electron Microscopy

Virus in suspension was spotted on formvar-coated carbonized grids of 2-300 mesh and was allowed to adhere to the surface for 1 minute. The bulk of the suspension was removed with a piece of filter paper. The virions were then washed 3 times by adding a drop of distilled water to the grid and then removing it by blotting at the edge. Material from cytoplasmic extracts was treated similarly only the grid was washed 10 times with distilled water. Blotting was done from one side of the grid only to stream out the molecules into visible linear strands. Both types of sample were then stained with 2% phosphotungstic acid (pH 6.8) and after 1 minute the stain was blotted off the filter paper.

The grids were then observed and photographed on a Phillips EM 300 electron microscope.

RESULTS

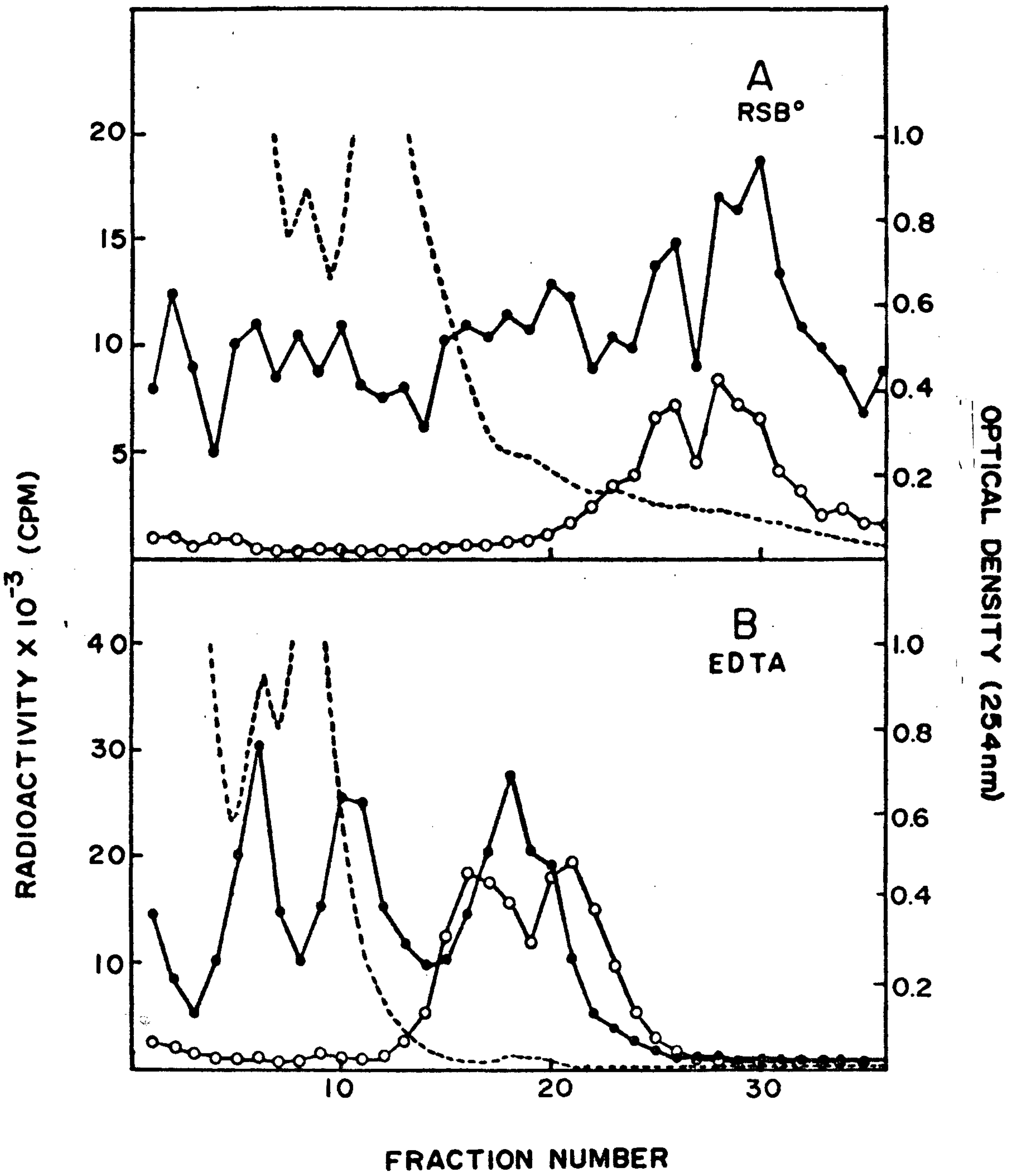
I. Viral RNA Species in the Cytoplasm of Cells During Infectious Virus Production

The experiments described in this section employed virus of the IND-HR-LT strain which had been plaque purified five times to ensure freedom from detectable amounts of defective particles in the stock. Under these conditions of infection the only detectable virions produced are the infectious B particles. Thus the RNA and ribonucleoprotein structures involved in the replication of infectious B particle virions can be observed without the added complexity of defective particle replication and interference.

In the first experiment VSV specific RNA present in the cytoplasm of infected cells was analyzed by sedimentation through a linear sucrose gradient in RSB° buffer. For this study infected cells were labelled with ^{3}H-uridine in the presence of Act D and cytoplasmic extracts prepared at 6 hours post-infection as described in METHODS. Analysis of this cytoplasmic extract yielded an optical density pattern showing the distribution of polyribosomes (fraction 16 to 30), monoribosomes (fraction 12), 50S ribosomal subunits (fraction

Figure 1. Cytoplasmic Extracts of Cells During Infectious Virus Production

Cells (5×10^7), infected with plaque purified virus at an M.O.I. of 50 PFU/cell, were exposed to ^{3}H-uridine from 3 to 6 hours post-infection. The infected cells were harvested and cytoplasmic extracts prepared as described in METHODS. Material containing subviral ribonucleoprotein with sedimentation coefficients of 140S and 160S labelled with ^{14}C-amino acids (20,000 cpm) obtained from Chil Yong Kang was placed on 15 to 30% linear sucrose gradients in RSB° buffer in (A) and EDTA buffer in (B) along with the cytoplasmic extracts of the infected cells. The gradients were then centrifuged at 81,000 x g for 4 hours. The optical density (254 nm) (------------) was continuously monitored as 1 ml fractions were collected. A 0.2 ml portion of each fraction was acid precipitated on nitrocellulose filters and the radioactivity of ^{3}H-uridine (●—●—●—●—●—●) and ^{14}C-amino acids (○—○—○—○) determined.


A
RSB°
B
EDTA
RADIOACTIVITY X 10^{-3} (CPM)
OPTICAL DENSITY (254nm)
FRACTION NUMBER
20
15
10
5
40
30
20
10
1.0
0.8
0.6
0.4
0.2
10
20
30

8), and 30S ribosomal subunits (fraction 6) presented in Figure 1A. The distribution of virus-specific RNA is indicated by the heterogeneous distribution of ^{3}H-uridine radioactivity found throughout the gradient.

The subviral ribonucleoprotein (RNP) sedimented to a position indicated by the ^{14}C-amino acid labelled marker (fractions 25 and 28). This material has been shown to contain RNA and protein bound in structures described as subviral ribonucleoprotein. From this previous work (Petric and Prevec, 1970) it seems likely that a large proportion of RNA sedimenting in the polyribosome region is viral messenger RNA associated with cosedimenting polyribosomes.

In an effort to examine the RNA of the subviral ribonucleoprotein in the absence of polyribosome-associated RNA, the cytoplasmic extract was centrifuged through a sucrose gradient containing only Tris-EDTA buffer. Under these conditions of centrifugation, as shown in the optical density profile of Figure 1B, the polyribosomes and monoribosomes have dissociated to yield ribosomal subunits. These subunits have sedimentation coefficients of 30S and 50S in EDTA buffer (Huang et al., 1970). At fraction 18 there is a small peak in the optical density profile. Associated with this material is the ^{14}C-amino acid labelled marker of subviral ribonucleoprotein. Under these gradient conditions there are now three definite peaks in the distribution of ^{3}H-uridine labelled RNA. One peak containing some 37% of the labelled viral RNA sediments with the subviral ribonucleoprotein. The other two peaks coincide with the 30S ribosomal subunit and the 50S ribosomal subunit and contain 23% and 27% of the labelled RNA respectively.

From the previous work of Huang et al. (1970) and Mudd and Summers (1970) as described in the INTRODUCTION, it seemed likely that the RNA in the two light ribonucleoprotein peaks might contain predominantly messenger RNA species. The peak coincident with the subviral ribonucleoprotein marker might contain those RNA species involved in either replication or assembly of subviral structures. These should be much easier to discern as the bulk of the transcription products were removed with the dissociation of the polyribosomes. To substantiate the above hypotheses RNA in these 3 peaks was extracted and characterized by both sedimentation on linear sucrose gradients in STE-SDS buffer and hybridization to RNA from infectious B particle virions.

A. Characterization of RNA from the Gradient Fractions Coincident with the 30S and 50S Ribosomal Subunits

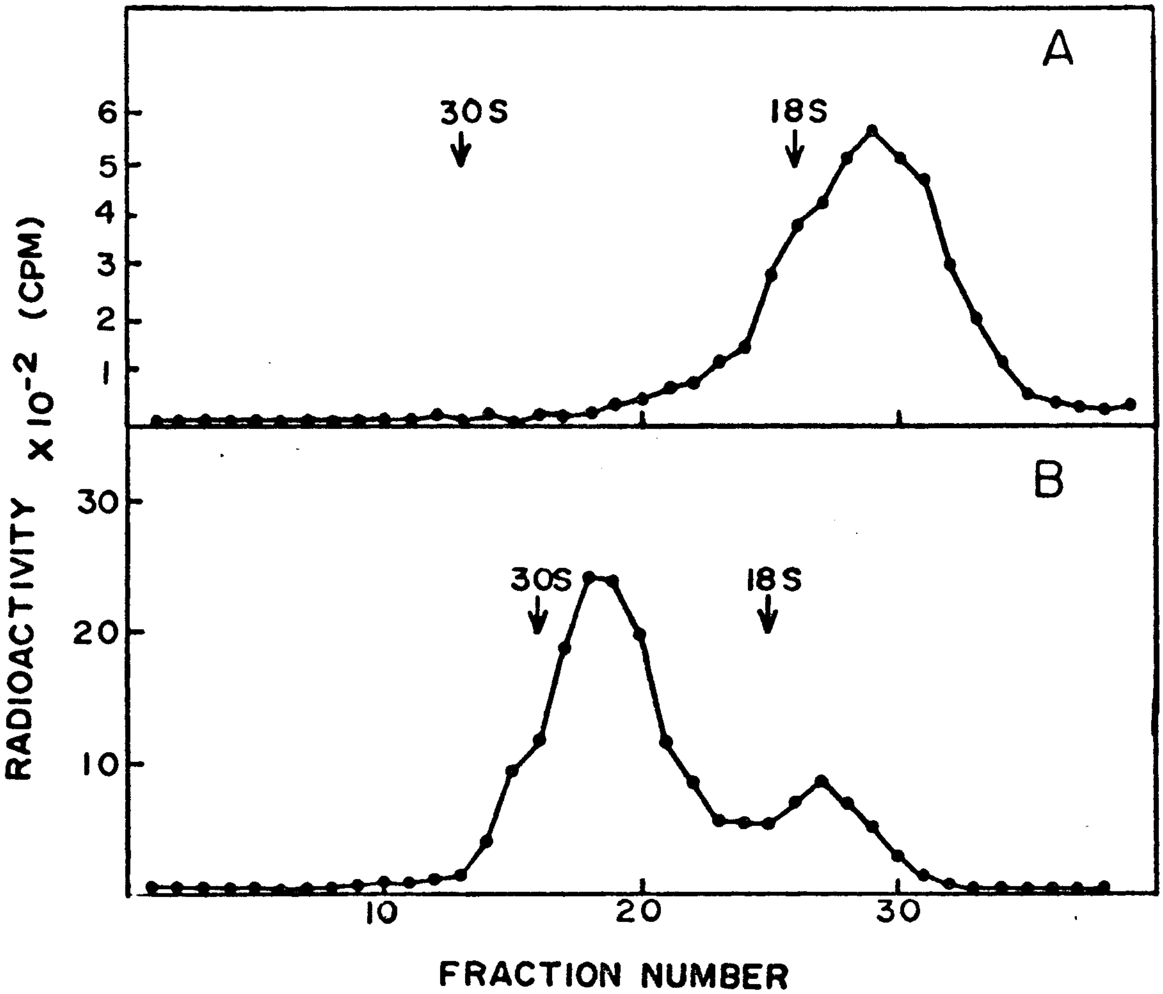
The sucrose gradient analysis of RNA, phenol extracted from the material coincident with the 30S ribosomal subunit, is shown in Figure 2A. The only labelled RNA species sediment in a broad distribution at about 15S.

Figure 2. Messenger-like Viral RNA Species Coincident with the 30S and 50S Regions

The RNA was extracted from fractions 5, 6 and 7 and 9, 10 and 11 of the gradient shown in Figure 1B and layered on individual linear sucrose gradients in STE-SDS buffer. ^{14}C-uridine labelled ribosomal RNA marker was also added (4000 cpm). The gradients were centrifuged at 185,000 x g for 2 hours. The gradients were collected by the dripping procedure. ^{3}H-uridine labelled radioactivity was plotted (●—●—●—●) and ^{14}C-uridine marker peaks were labelled with their sedimentation coefficients at 30S and 18S.

(A) RNA extracted from the 30S region.

(B) RNA extracted from the 50S region.


A
30S
18S
B
30S
18S
6
5
4
3
2
1
30
20
10
RADIOACTIVITY x 10^-2 (CPM)
10
20
30
FRACTION NUMBER

RNA extracted from the material coincident with the 50S ribosomal subunits showed two peaks in the radioactive distribution when sedimented in a linear sucrose gradient (Figure 2B). One peak was a sedimentation coefficient of 27S and the smaller second peak a sedimentation coefficient of approximately 15S. It is possible that the small amount of 15S RNA in this fraction results in the incomplete separation of the 50S and 30S material in the cytoplasmic gradients.

These results were similar to those of Huang et al. (1970) who defined the 27S and 15S species of RNA as messengers. If only complementary RNA species (i.e., RNA species which can form double stranded hybrids with virion RNA) are present in these fractions it should then be possible to obtain complete hybridization of this RNA with viral RNA. To check this, RNA species from fractions coinciding with the 30S and 50S region of a gradient as shown in Figure 1B were extracted for annealing to B particle virion RNA.

The RNA from both regions was largely single-stranded after phenol extraction as only 5.7% of the RNA extracted from the 30S region and 5.8% of the RNA from the 50S region was resistant to pancreatic ribonuclease digestion. After self-annealing in the absence of any added B particle RNA, the RNA from the 30S region showed 8.3% and the RNA from the 50S region 16.0% resistance to ribonuclease digestion. Thus

Figure 3. Hybridization of mRNA to Infective B Particle Virion RNA

^{3}H-uridine labelled RNA from the 30S region (A) and 50S region (B) of gradients similar to that shown in Figure 1B was extracted. Samples of this RNA (4000 cpm) were then allowed to anneal with 1, 2 and 5 μg of unlabelled RNA extracted from infective virions. These samples were then divided into two equal portions and one was exposed to digestion with pancreatic ribonuclease (50 μg/ml). Both portions were then acid precipitated and the radioactivity counted. Percent RNA hybridized =

$$\frac{\text{acid precipitable RNA after digestion (cpm)}}{\text{acid precipitable RNA before digestion (cpm)}} \times 100$$

was plotted (●—●—●—●—●). The amount of self-annealing of the labelled RNA was also determined by a similar method except that no virion RNA was added.

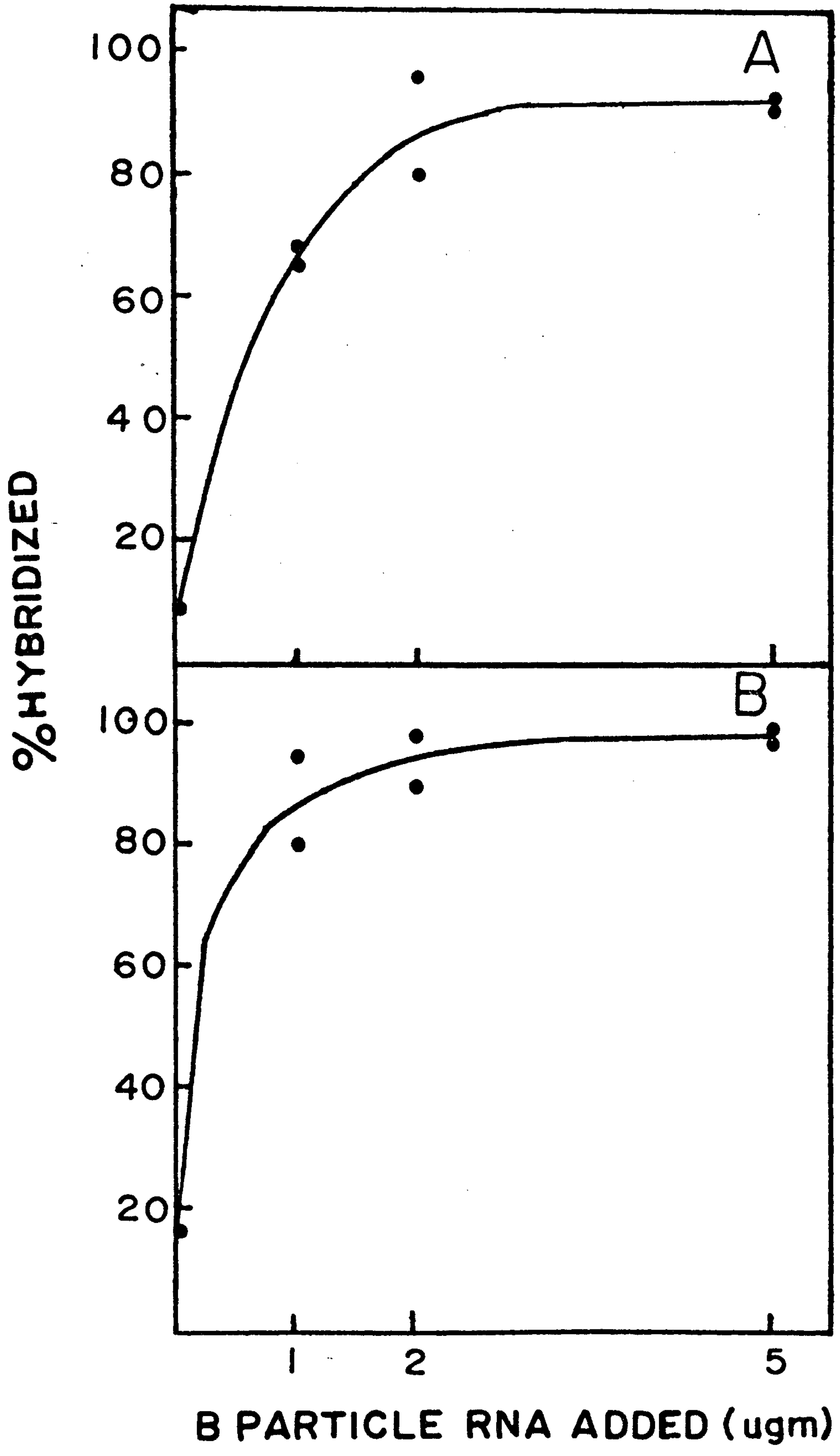

A
B
100
80
60
40
20
% HYBRIDIZED
1
2
5
B PARTICLE RNA ADDED (ugm)

Table 1

Labelled RNA was extracted from the peaks coincident with the 50S and 30S ribosomal subunits in a gradient similar to that shown in Figure 1B. This RNA was separated into 27S and 15S species on linear sucrose gradients by an experiment similar to that described in Figure 2. After collecting 0.25 ml fractions of the gradient using an Isco fractionator, 50 μl samples were removed from each fraction and after acid precipitation the radioactivity was monitored. The fractions containing labelled 27S and 15S RNA were separately pooled and the RNA precipitated from the fractions with ethanol. The RNA was redissolved in Tris buffer and then annealed either in the absence of any added RNA or in the presence of 4 μgm of B particle RNA under the conditions defined in METHODS. Approximately (4000 cpm) of 15S RNA and 27S RNA was used in each reaction. Each sample was split into two portions, one portion acid precipitated directly while the other was treated with ribonuclease as described in Figure 3. The percentage of RNA nuclease-resistant compared to the total radioactivity is presented in the Table.

Table 1

Annealing of Messenger RNA with RNA Extracted from Infectious B Particles

Labelled Messenger Species	Percent Ribonuclease Resistance	
	No Added RNA	4 μgm B Particle
15S	5	90
27S	28	93

some complementary RNA species were present in these fractions. As seen in Figures 3A and 3B, the RNA from both the 30S and 50S region hybridized completely in the presence of excess unlabelled virion RNA. Thus it would appear that virtually all of the RNA in these fractions is complementary to virion RNA in agreement with the observation of Huang *et al*. (1970).

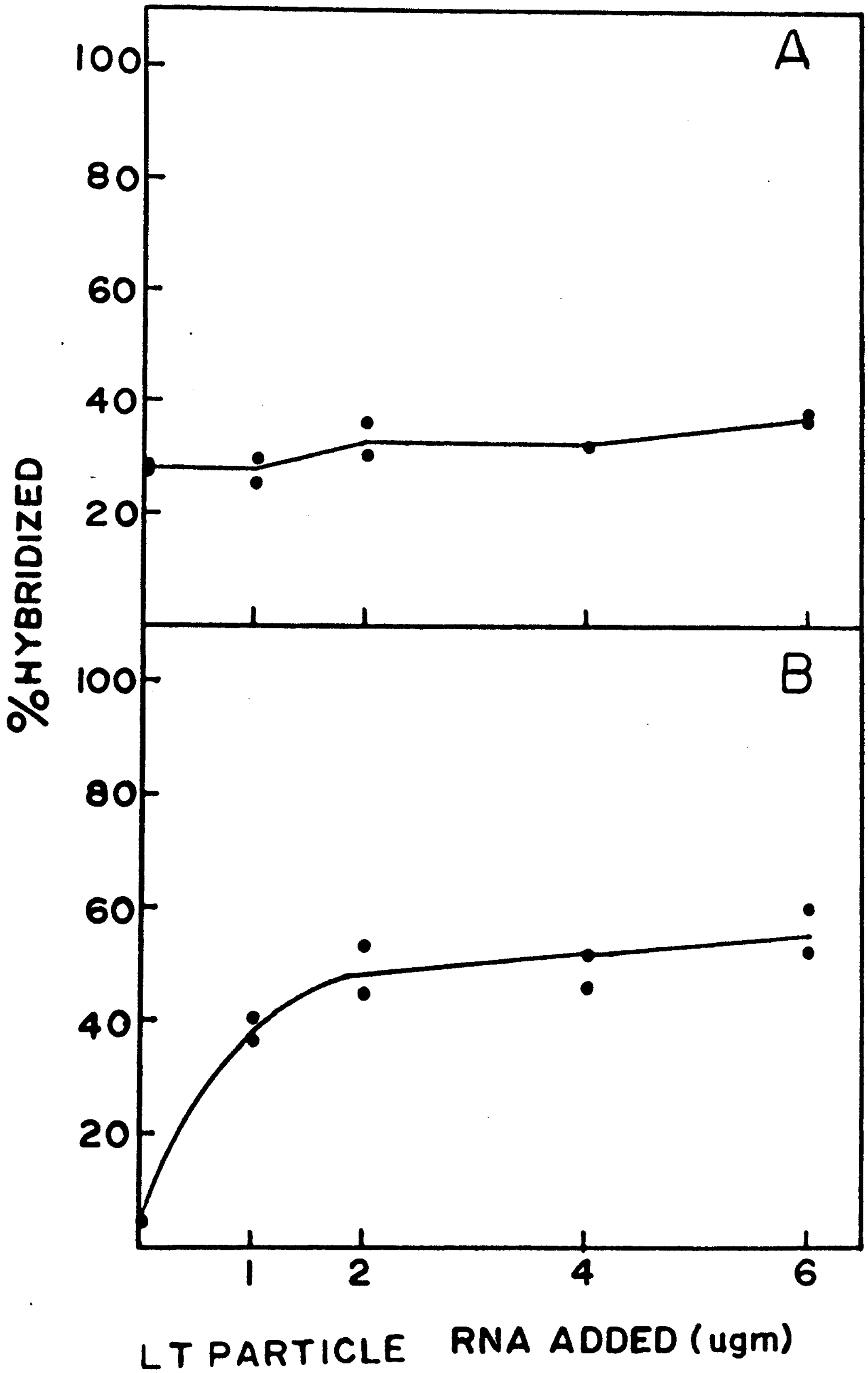
Figure 2B showed that the RNA extracted from the 50S region contained a small proportion of 15S RNA as well as 27S RNA. In order to examine the hybridization properties of the 27S RNA alone, these species were separated on linear sucrose gradients. In this case the purified 28S RNA species and the 15S RNA species both hybridized to over 90% with excess B particle virion RNA as shown in Table 1. More interesting, however, is the fact that the 27S species of RNA showed 28% resistance when allowed to self-anneal. This self-annealing is indicative of both positive and negative strands in this RNA species and was not found by Huang *et al*. (1970). This will be discussed later.

B. Hybridization of 27S and 15S Messenger-RNA Species with LT Particle Virion RNA

As seen above the 15S and 27S messenger species of RNA produced in the cytoplasm of cells infected under conditions of minimal interference hybridize 100% to B particle virion RNA. It was of some interest to examine the degree of homology

Figure 4. Hybridization of 28S and 15S Messenger RNA
Species of LT particle Virion RNA

^{3}H-uridine labelled RNA was extracted from the regions of a gradient as shown in Figure 1B corresponding to the 50S and 30S ribosomal subunits. This RNA was separated into 28S and 18S species as described in METHODS Section 12. Samples of this RNA were allowed to anneal with 1, 2, 4 and 6 μg of unlabelled RNA extracted from defective LT particle virions. These samples were then divided into two equal portions and one portion was exposed to digestion with pancreatic ribonuclease (50 μg/ml). Both portions were then acid precipitated and the radioactivity counted. Percent hybridization was plotted (●—●—●). The amount of self-annealing of the radioactive RNA was also determined by a similar method except that no virion RNA was added.


A
B
100
80
60
40
20
%HYBRIDIZED
1
2
4
6
LT PARTICLE RNA ADDED (ugm)

existing between these complementary RNA species and the RNA genome of the LT defective particle.

The LT particle virions, from which RNA was extracted were taken through two successive gradient purifications to ensure greater freedom from contamination with infectious B virions.

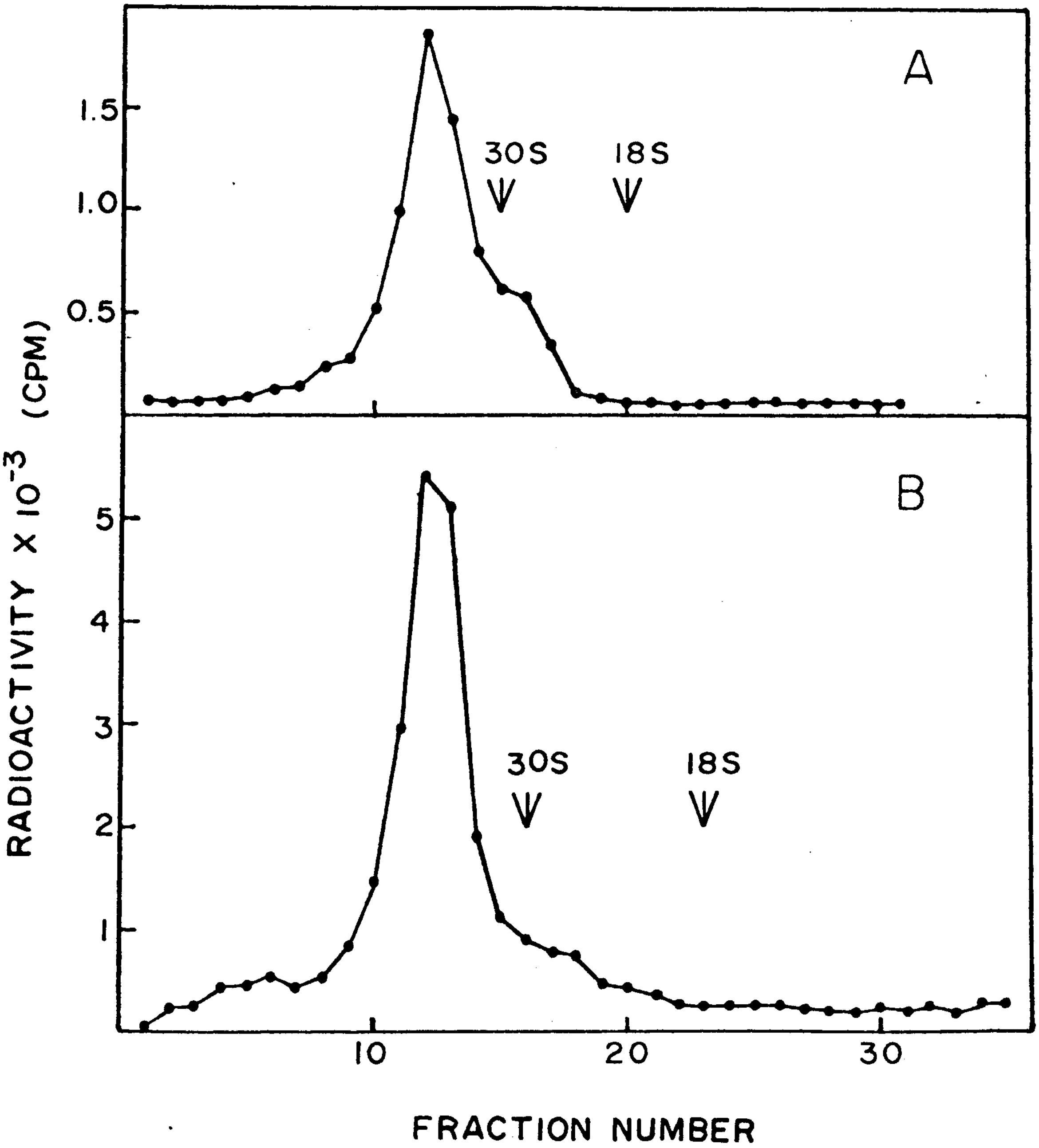
Both the 15S and 27S species of RNA, after separation on the basis of their sedimentation coefficients on linear sucrose gradients, were recollected and annealed to various amounts of LT virion RNA as shown in Figure 4. Figure 4B shows that 55% of the 15S species of messenger RNA annealed to LT virion RNA. This same 15S species of RNA showed only 4% self-annealing. Figure 4A shows that the 27S messenger RNA showed 38% resistance to digestion with pancreatic ribonuclease when allowed to anneal in the presence of up to 6 μg of LT virion RNA. However this same 28S RNA species was shown to self-anneal 29%. Therefore the 15S messenger species has base sequences 55% complementary to LT virion RNA and the 27S messenger species contains no base sequences complementary to LT virion RNA.

C. Characterization of the RNA from the Heavy Complex Coincident with the Subviral Ribonucleoprotein Marker

For these studies RNA was extracted from the fractions of the peak coincident with ^{14}C-labelled subviral ribonucleoprotein

Figure 5. Comparison of the RNA Extracted from the RNP Region and Infectious Virions

RNA was extracted from fractions 17, 18 and 19 in Figure 1B in (A) and from ^{3}H-uridine labelled infective B particle virions in (B). This RNA was layered on individual linear sucrose gradients in STE-SDS buffer. The gradients were centrifuged at 107,000 x g for 3 hours and collected by the dripping procedure. ^{3}H-uridine labelled radioactivity (●—●—●—●) and ^{14}C activity of added ribosomal marker RNA were determined and plotted. The sedimentation coefficients of peaks in the ^{3}H-uridine distribution were extrapolated from the values of 30S and 18S assigned to the rRNA of the marker.

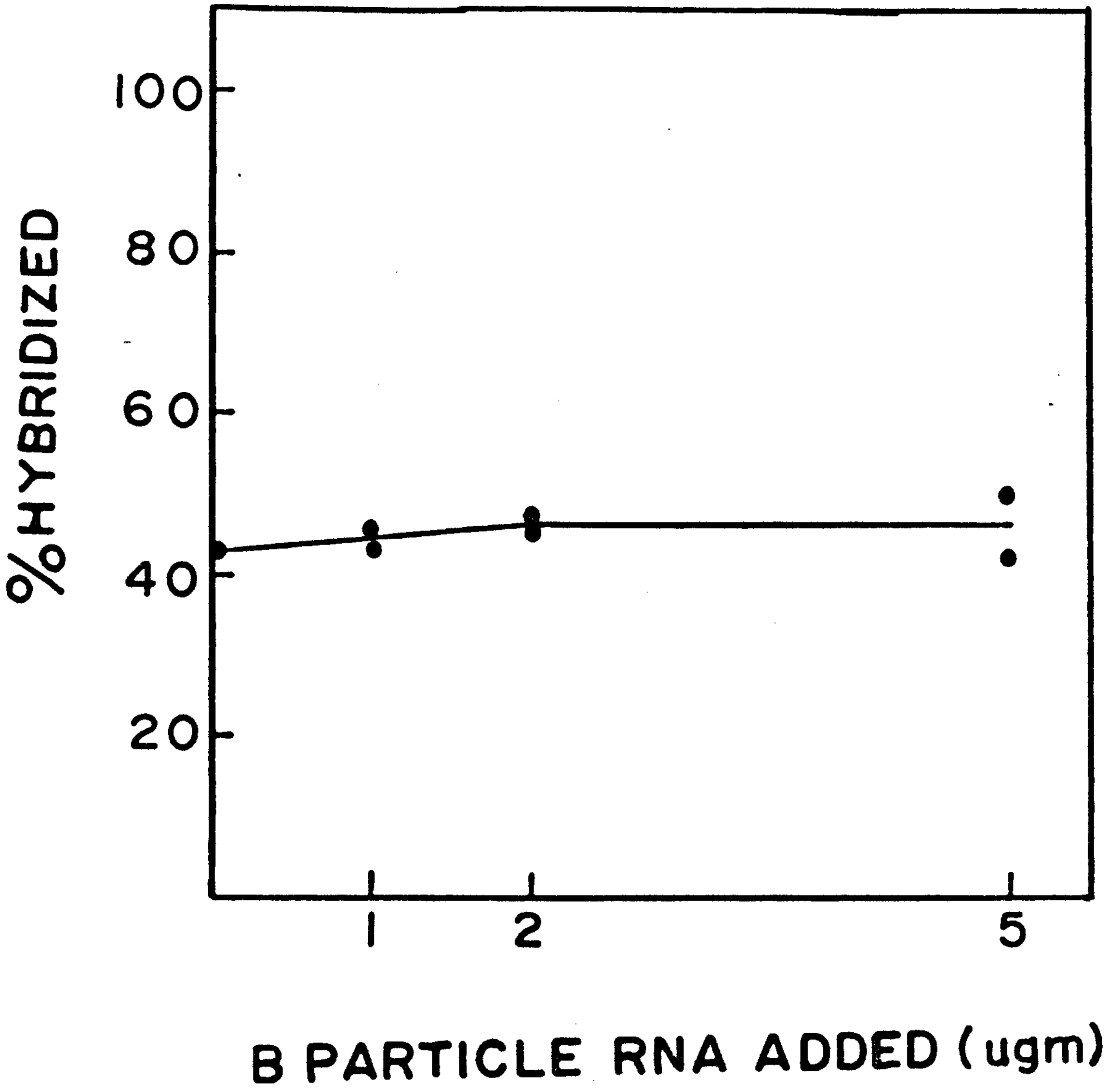

A
B
30S
18S
30S
18S
1.5
1.0
0.5
5
4
3
2
1
10
20
30
RADIOACTIVITY X 10^{-3} (CPM)
FRACTION NUMBER

marker as in the gradient shown in Figure 1B. As seen in Figure 5A, the majority of the labelled RNA from this fraction has a sedimentation coefficient of 37.5S. Some material of sedimentation coefficient 30S is also present. It seems unlikely that this represents RNA resulting from incomplete separation of the 30S RNA species found coincident with the 50S ribosomal subunit. As shown later this RNA species is more likely a distinct viral product. The sedimentation coefficient of 37.5S seemed characteristic of B particle virion RNA. To check this labelled RNA extracted from B particle virions was centrifuged under similar conditions. As seen in Figure 5B the sedimentation coefficient of this RNA is indeed 37.5S.

In light of this similarity the RNA from the heavy complex was further characterized by annealing to unlabelled RNA extracted from the B particle virions. The RNA was completely single-stranded after extraction as it showed only 2.7% resistance to digestion with pancreatic ribonuclease. As seen in Figure 6, 40% of the radioactive RNA extracted from the heavy complex was resistant to ribonuclease digestion after annealing to unlabelled virion RNA. However this radioactive RNA showed 43% self-annealing in the absence of any unlabelled virion RNA. The amount of annealing did not therefore increase significantly with the addition of excess virion RNA.

Figure 6. Hybridization of RNA Extracted from Subviral RNP Complex to RNA of Infective B Particle Virions

RNA was extracted from the fractions containing the RNP-like complex in a gradient similar to that in Figure 1B. Samples of this RNA (4000 cpm) were then annealed with 1, 2 and 5 μg of unlabelled RNA extracted from infective virions as described in METHODS. Each sample was then divided into two equal portions and one portion was treated with pancreatic ribonuclease (50 μg/ml). Both portions were then acid precipitated and the radioactivity counted. Percent RNA hybridized was then plotted (●—●—●—●). The amount of self-annealing of the radioactivity labelled RNA was determined by the sample with no added virion RNA.


% HYBRIDIZED
100
80
60
40
20
1
2
5
B PARTICLE RNA ADDED (ugm)

This result indicates that substantial amounts of complementary RNA are present in the RNA extracted from the heavy complex. Since 57% of the RNA remains unhybridized in the presence of excess virion RNA and since the majority (approximately 70%) of the RNA has a sedimentation coefficient of 37.5S, it is concluded that the major labelled species in this fraction is newly synthesized virion RNA. If one assumes equal labelling of all viral RNA species in the complex then some 23% of the RNA in the heavy complex may be messenger-like or complementary to the B particle genome. As approximately 30% of the labelled RNA has a sedimentation coefficient between 37.5S and 30S the exact size of these complementary strands is unknown. The possible significance of these complementary strands will be discussed later.

D. Demonstration that the Heavy Complex is a Ribonucleoprotein Complex

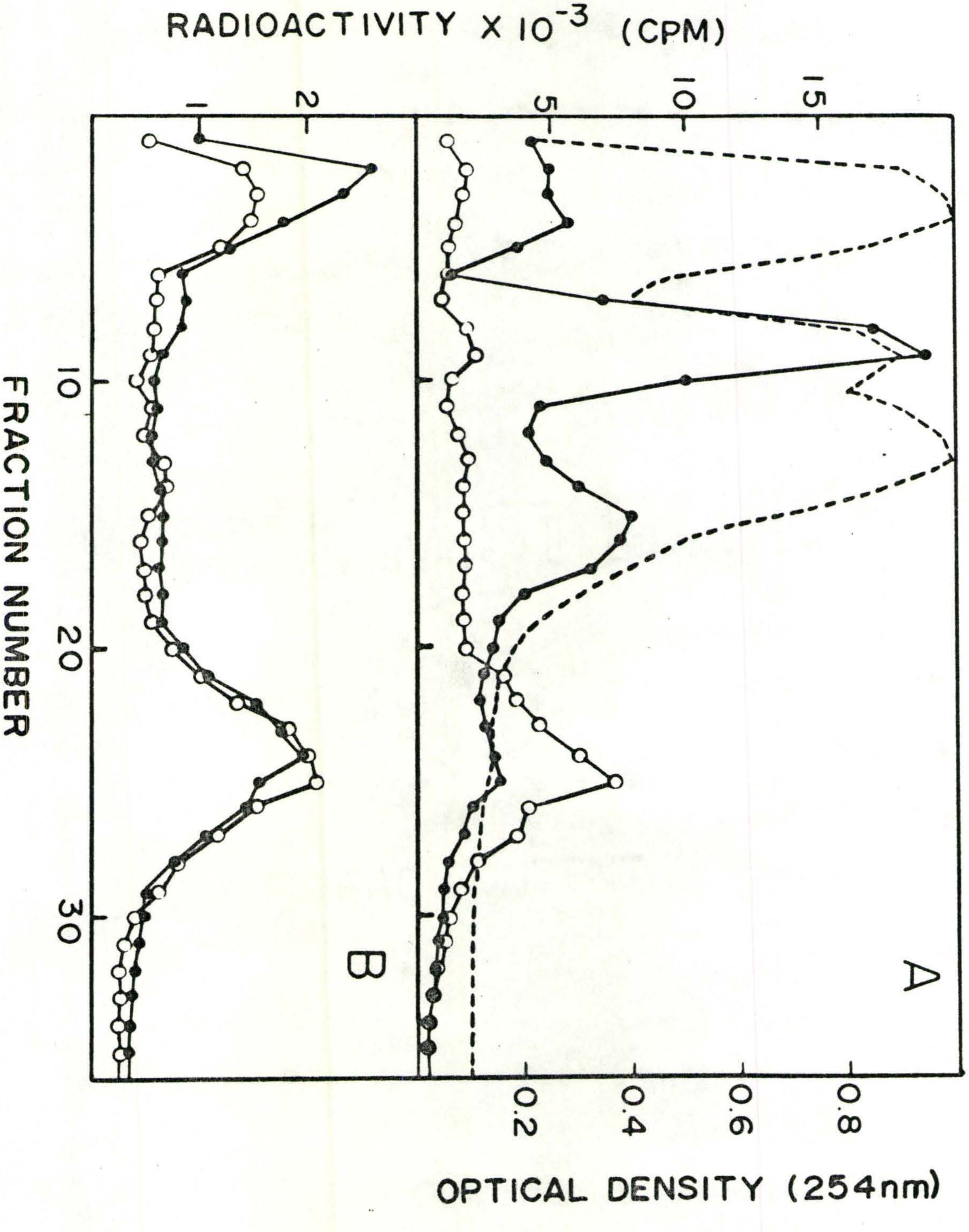
To demonstrate that the heavy complex present in EDTA gradients was a subviral ribonucleprotein complex, it was necessary to show that protein was associated with the viral RNA and that this protein protected, at least in part, the viral RNA from nuclease digestion (Wagner _et al._, 1969). Infected cells were labelled with both ^{14}C-amino acids and ^{3}H-uridine as described in Figure 7A. Analyses of the cytoplasmic extract on a sucrose gradient show a peak of ^{14}C-amino acid

Figure 7. Resistance of a Cytoplasmic Extract to Digestion with Pancreatic Ribonuclease

Cells (5×10^7) were exposed to ^{3}H-uridine and ^{14}C-amino acids from 3 to 6 hours post-infection at an M.O.I. of 50. An IND-HR stock which was plaque purified five times to minimize the presence of defective virus particles was used. The cells were harvested at 6 hours post-infection and the cytoplasmic extract layered on a linear sucrose gradient in EDTA buffer. The gradient was centrifuged at 81,000 x g for 6 hours. The optical density (254 nm) (-------------) was continuously monitored as 1 ml fractions were collected. Two 0.1 ml portions of each fraction were taken and one treated with pancreatic ribonuclease (5 μg/ml) for 30 minutes at 37°C. Both portions were then acid precipitated on nitrocellulose filter and the radioactivity of ^{3}H-uridine (●—●—●—●) and ^{14}C-amino acids (○—○—○) monitored.

(A) before ribonuclease digestion.

(B) with ribonuclease digestion.


RADIOACTIVITY X 10^-3 (CPM)
15
10
5
2
1
A
B
FRACTION NUMBER
10
20
30
OPTICAL DENSITY (254nm)
0.2
0.4
0.6
0.8

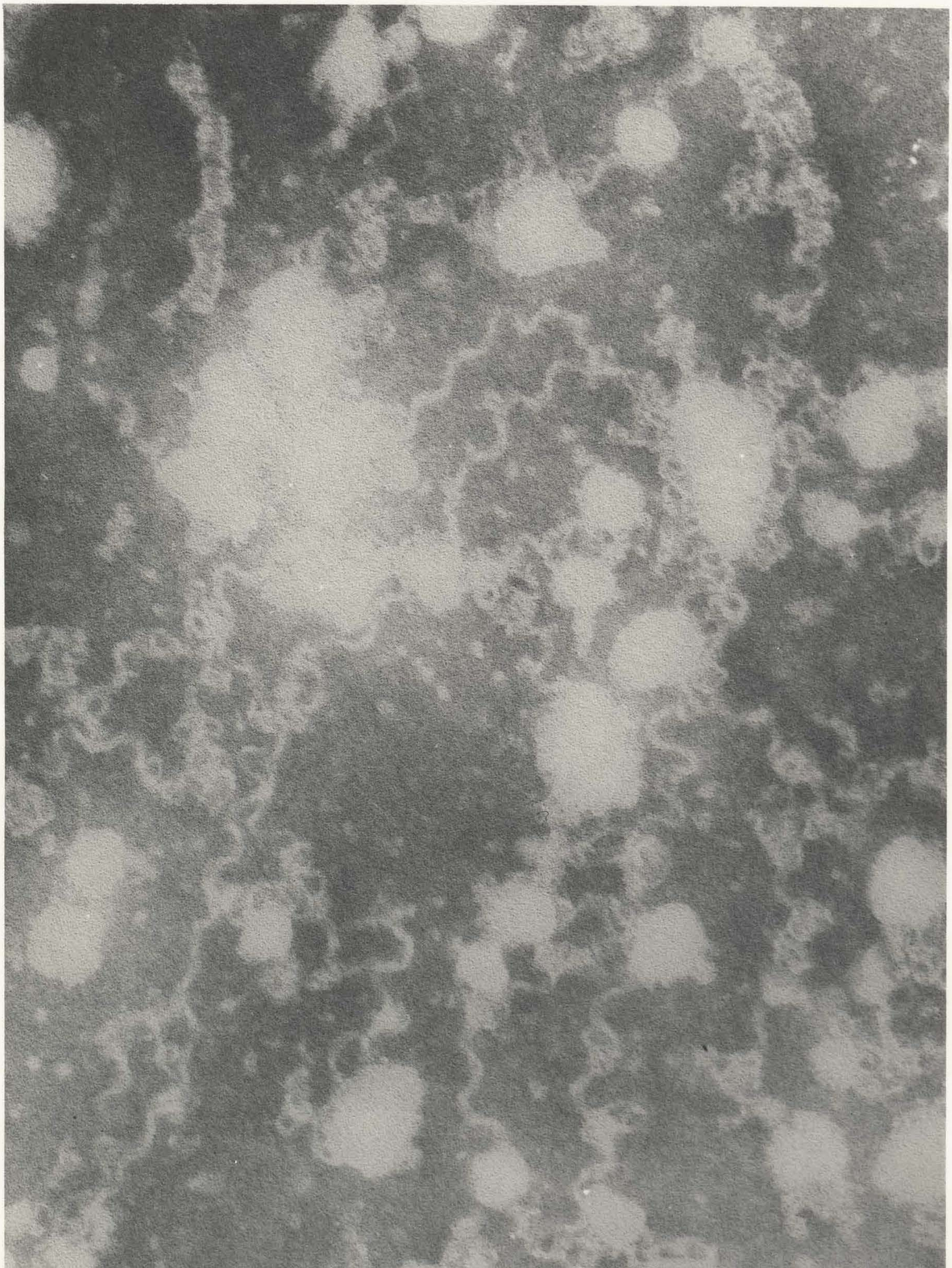
labelled material coincident with the ^{3}H-uridine of the heavy complex.

When a portion of each fraction of the gradient shown in Figure 7A was digested with pancreatic ribonuclease the only ^{3}H-uridine labelled material which remained acid-precipitable was that present in the heavy complex (Figure 7B). Approximately 66% of the RNA in this fraction was recovered. RNA, phenol extracted from this complex, has been shown to be single-stranded. The resistance to nuclease in this experiment must therefore be due to tight binding of the protein molecules to the RNA molecules forming an RNP complex.

Further evidence for the existence of subviral-like RNA complex comes from electron micrographs of material obtained from this region of the gradient. As seen in Figure 8, long RNP structures with helical symmetry, identical in appearance to RNP complexes isolated from virus particles (Nakai and Howatson, 1968) are observed.

Figure 8. Electron Micrograph of Material Taken from Fractions Containing the RNP Complex Found in EDTA Gradients

Fractions were taken from the peak corresponding to the RNP complex similar to that shown in Figure 7. A small sample was spotted on a Formvar-covered copper grid and stained with phosphotungstic acid as described in METHODS. The magnification is X300,000. Bar represents 0.1 nm.



II. RNA Sequences in the Cytoplasm of Cells Infected Under Conditions of Defective Particle Production

The above studies examine the ribonucleoprotein complex present in cells infected under conditions in which little or no defective virus particle replication is occurring. The remainder of this work uses EDTA gradients to examine the ribonucleprotein components present in cells infected with and producing defective particles. The first experiment serves to describe the characteristics of the defective particle containing stocks used in subsequent experiments.

Two stocks were prepared as described in METHODS. One, designated HR-LT produces 50% defective LT particles and 50% infectious B particles, and the other, designated IND-ST produces predominantly ST particles. Figure 9A shows the optical density pattern of a linear sucrose gradient containing virus material produced by the HR-LT stock. The two peaks of optical density (mainly light scattering) in the pattern are characteristic of B and LT virions as indicated. Figure 9B shows the optical density pattern of a linear sucrose gradient containing virus material produced by the IND-ST stock. The one major peak in the pattern is characteristic of the ST virions which have a lower sedimentation coefficient than LT or B particle virions.

Figure 9. Sedimentation Characteristics of B, ST and LT Particle Virions on Linear Sucrose Gradients

Cells (10^8) were infected at an M.O.I. of 20 with 2 stocks, HR-LT in (A) and IND-ST (plus an equal amount of IND-ST stock containing predominantly ST particles) in (B). ^{3}H-uridine was added at 3 hours post-infection and the virus harvested at 16 hours post-infection. The harvested virus was placed on 5 to 30% linear sucrose gradients in PBS and centrifuged for 35 minutes at 81,000 x g. The optical density (254 nm) was continuously monitored as 1 ml fractions were collected (------------).

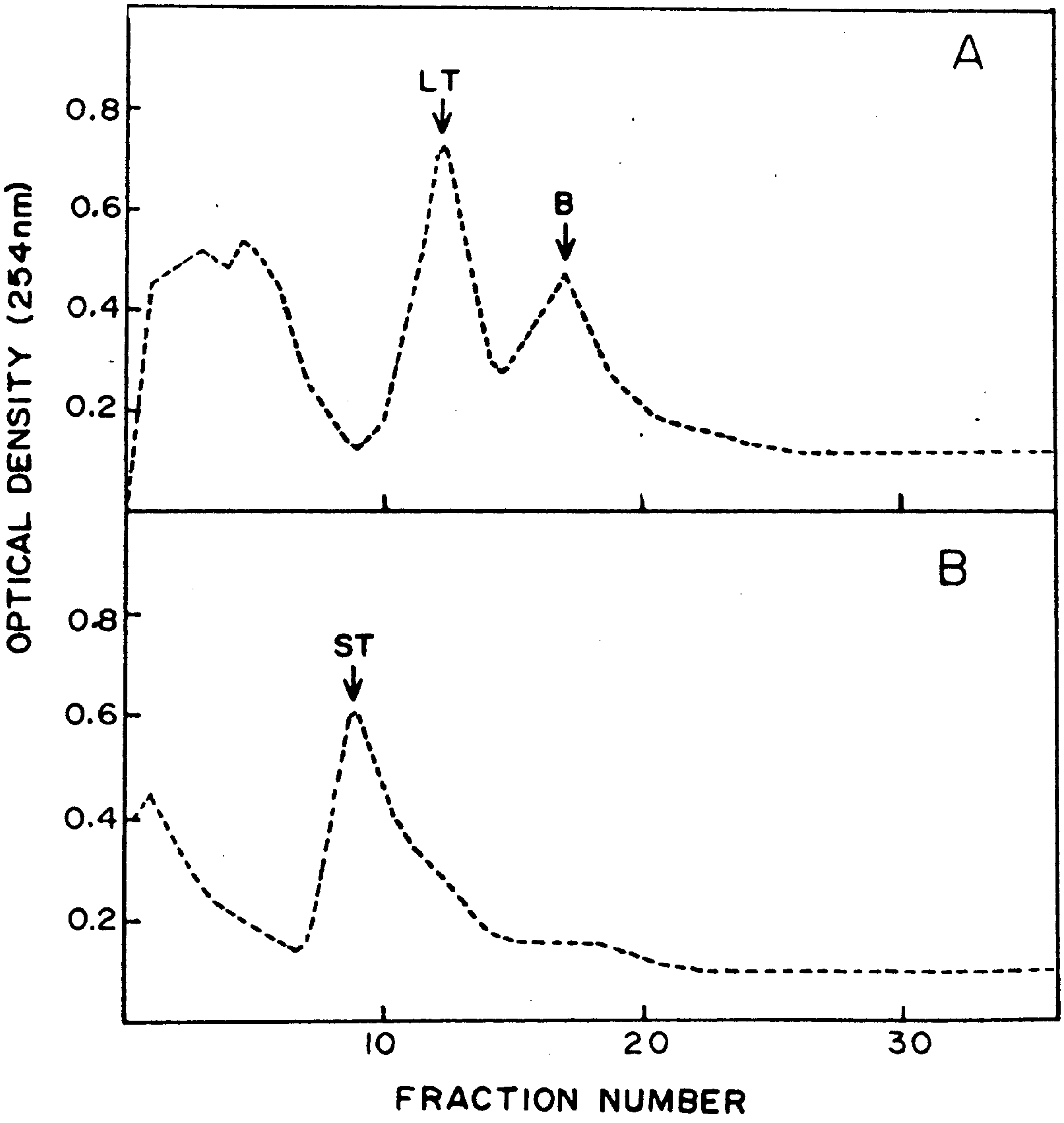

A
B
LT
B
ST
OPTICAL DENSITY (254nm)
0.8
0.6
0.4
0.2
10
20
30
FRACTION NUMBER

Figure 10. RNA Extracted from LT and ST Virions

Fractions 11, 12 and 13, from the gradient in Figure 9A (corresponding to LT virions) and fractions 8, 9 and 10 from the gradient in Figure 9B were pooled and the RNA was extracted. This ^{3}H-uridine labelled RNA was placed on linear sucrose gradients in STE-SDS buffer along with ^{14}C-uridine labelled ribosomal RNA marker (4000 cpm). The gradients were centrifuged at 107,000 x g for 3 hours. The material was collected by dripping and the radioactivity monitored. ^{3}H-uridine radioactivity was plotted (●—●—●—●) and ^{14}C-uridine labelled marker peaks designated with their characteristic sedimentation coefficients at 30S and 18S. The sedimentation coefficients of the ^{3}H-uridine labelled peaks extrapolated from the marker values are also shown.

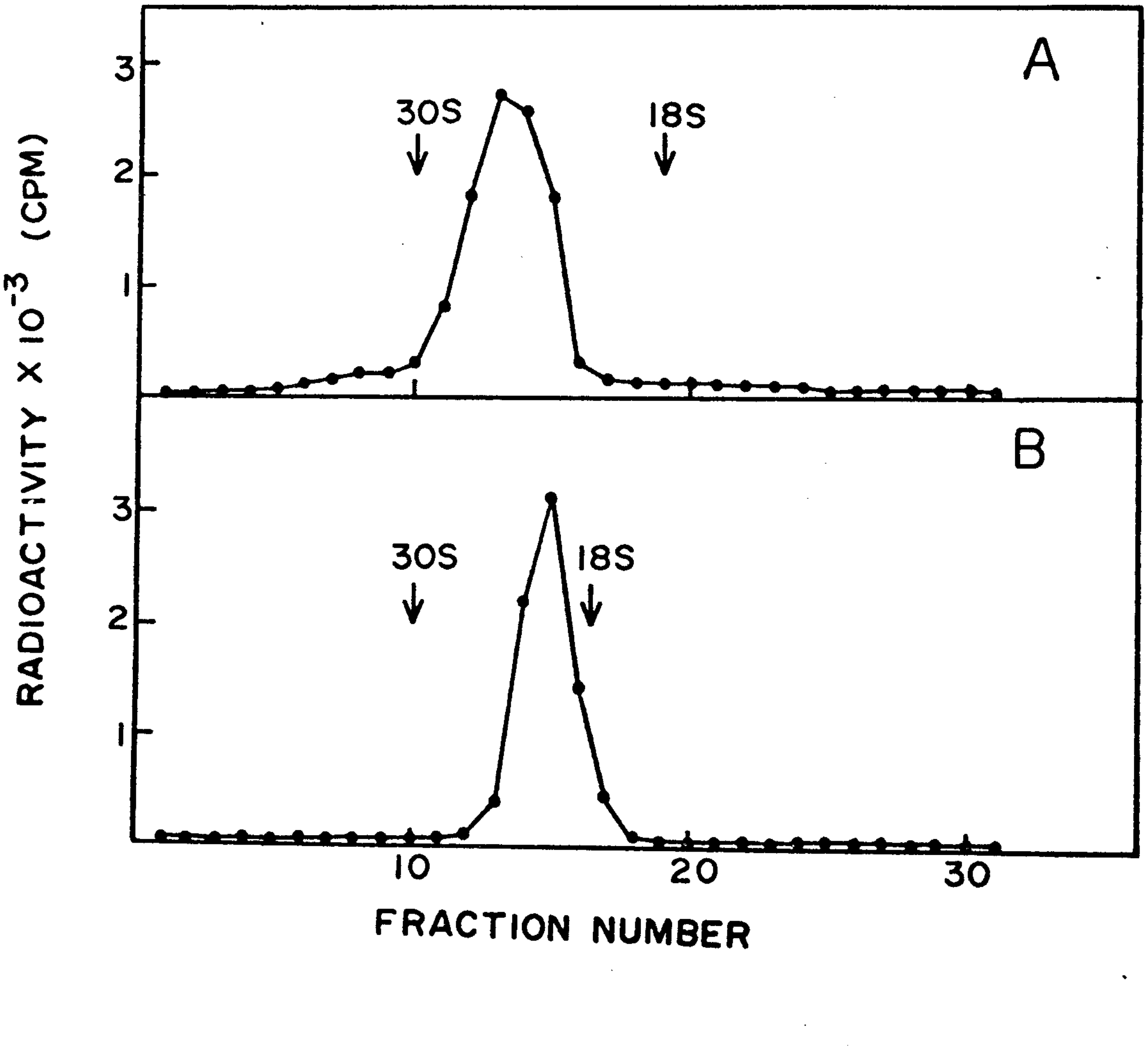
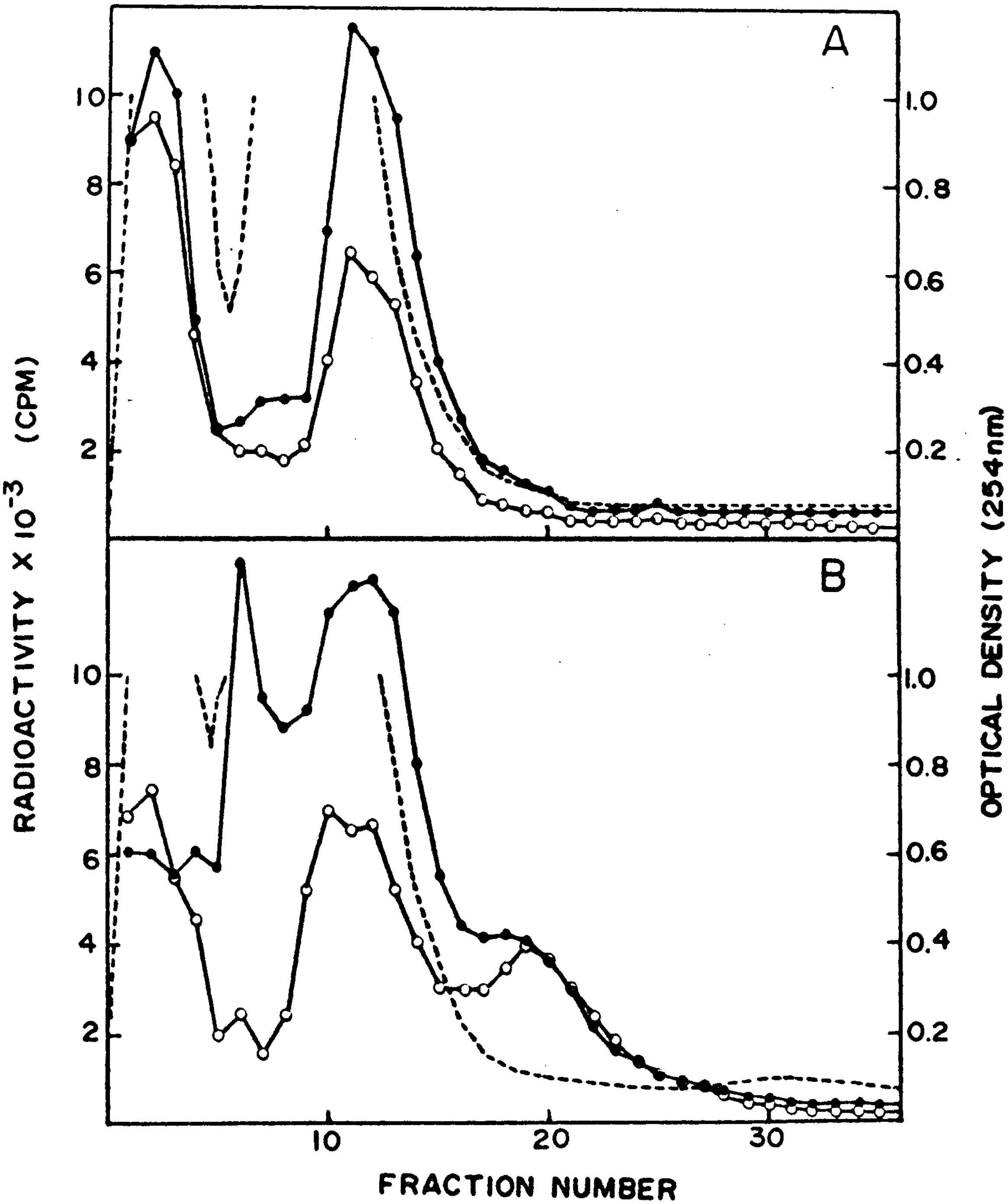

A
30S
18S
B
30S
18S
3
2
1
3
2
1
RADIOACTIVITY X 10^{-3} (CPM)
10
20
30
FRACTION NUMBER

Figure 11. Cytoplasmic Extract of Cells Infected Under ST Particle Interference Conditions

Cells (5×10^7) were infected with B particle stock at an M.O.I. of 50 and an equal volume of IND-ST stock containing predominately ST particles in (A), and with B particle stock of an M.O.I. of 50 with one-twenty-fifth the volume of IND-ST stock (B). ^{3}H-uridine and ^{14}C-amino acids were added at 3 hours post-infection and the cells were harvested at 6 hours post-infection. Cytoplasmic extracts were prepared and layered on linear sucrose gradients in EDTA buffer. The gradients were spun at 81,000 x g for 5 hours. The optical density (254 nm) was continuously (----------) monitored as 1 ml fractions were collected. A 0.2 ml portion of each fraction was acid precipitated and the radioactivity measured: ^{3}H-uridine (●—●—●—●) and ^{14}C-amino acid (○—○—○) radioactivity were plotted.


A
B
RADIOACTIVITY X 10^{-3} (CPM)
OPTICAL DENSITY (254nm)
FRACTION NUMBER
2
4
6
8
10
0.2
0.4
0.6
0.8
1.0
10
20
30

RNA was extracted from sucrose gradient purified LT and ST particles grown in the presence of ^{3}H-uridine. This RNA was analyzed by sucrose gradient sedimentation. As seen in Figure 10A, labelled RNA extracted from the LT virions shows one peak with a sedimentation coefficient of 26S. This sedimentation coefficient is in agreement with the value reported by Schincariol and Howatson (1970). Figure 10B similarly shows that RNA extracted from ST virions has a sedimentation coefficient of 21S. This is characteristic of the ST virions also used by Schincariol and Howatson (1970).

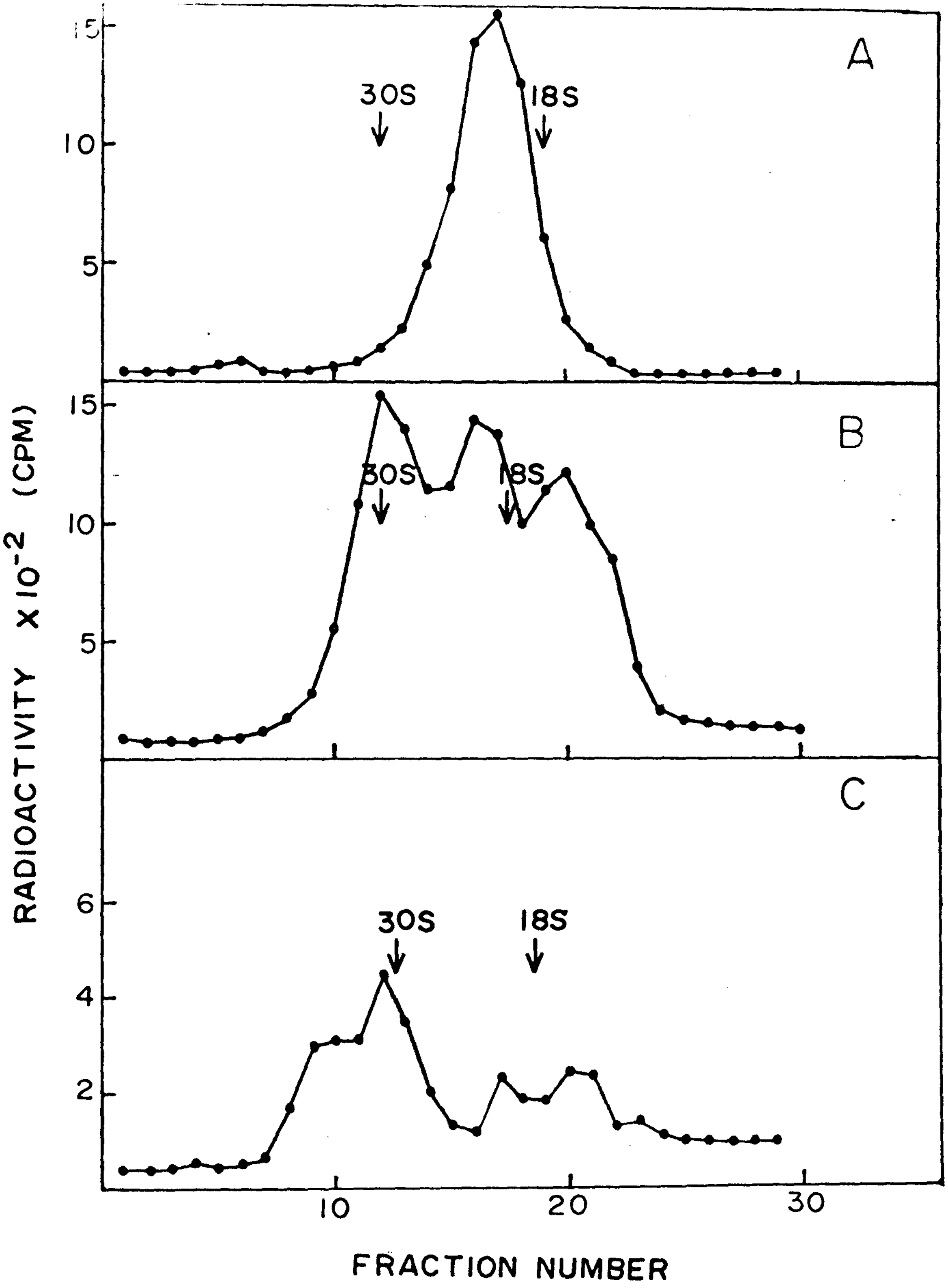
A. ST Particle Interference

Figure 11A shows the result of gradient analysis of ^{14}C-amino acid and ^{3}H-uridine labelled cytoplasmic extract of cells infected with a stock consisting of B particles mixed with an equal volume of the IND-ST stock described above. Infection under this condition results in a high level of interference and mainly ST particles are produced. ^{3}H-uridine and ^{14}C-amino acids show one coincident peak at fraction 11 in the gradient. This cosediments with the 50S ribosomal subunit region as shown by the O.D. tracing.

Figure 11B describes a similar experiment except that the cells were infected with a stock containing B particles mixed with one-twentyfifth the volume of IND-ST

Figure 12. RNA from RNP Complexes Found in Cells Infected Under ST Particle Interference Conditions

Fractions 11, 12 and 13 (A) from the gradient shown in Figure 11A; and fractions 10, 11 and 12 (B) and fractions 18, 19 and 20 (C) from the gradient shown in Figure 11B were separately pooled and the RNA extracted from each set of fractions. This ^{3}H-uridine labelled RNA was layered on linear sucrose gradients in STE-SDS buffer alone with the ^{15}C-uridine labelled ribosomal RNA marker (4000 cpm). The gradients were centrifuged at 107,000 x g for 3 hours, then collected by the dripping procedure, and the radioactivity counted. ^{3}H-uridine radioactivity was plotted (●—●—●—●) and ^{14}C-uridine marker peaks labelled at their characteristic sedimentation coefficients of 30S and 18S. The sedimentation coefficients of the peaks in the ^{3}H-uridine radioactivity distribution were extrapolated from the marker values.


A
B
C
30S
18S
15
10
5
6
4
2
RADIOACTIVITY X10^{-2} (CPM)
10
20
30
FRACTION NUMBER

stock used above. Under this condition of infection, as seen in Figure 11B, in addition to the peak at 50S there is another peak in both the ^{3}H-uridine and ^{14}C-amino acid distribution present at fractions 19 and 20. This peak has a sedimentation coefficient similar to that of the RNP peak described for cells infected in the absence of detectable defective particles.

In order to characterize the complexes observed under the two conditions of interference as described above, the RNA species from the peak fractions were phenol extracted and analyzed on sucrose gradients. Figure 12A shows the ^{3}H-uridine distribution of RNA extracted from the RNA complex of Figure 11A. The distribution shows a single peak having a sedimentation coefficient of 21S. This is characteristic of ST particle virion RNA as shown previously. Figure 12B shows the ^{3}H-uridine distribution of RNA extracted from the light peak fractions 10, 11 and 12 of Figure 11B. The distribution shows three peaks having sedimentation coefficients of 30S, 21S and 15S. The 21S peak is again characteristic of ST particle virion RNA. The 30S and 15S peaks are characteristic of the messenger species found coincident with the 50S ribosomal subunit region under conditions of infectious virus production. Figure 12C shows the ^{3}H-uridine distribution on a sucrose gradient of RNA extracted from the heavy peak fractions 18, 19 and 20 of the gradient shown in Figure 11B. It is evident that a number of RNA species are present. Major peaks are present at 37.5S and 32S along with small peaks at 21S and 15S. The large 37.5S species of RNA is similar to RNA extracted from the RNP's described previously. The 21S and 15S species were present in the lighter RNP peak described above. They are characteristic of ST virion RNA and messenger RNA respectively. The 32S species of RNA seen in this experiment has not been found as a major species of RNA described elsewhere in this thesis or in the literature. The RNP complex found under conditions of infectious virus production does contain species of RNA of this size but in minor amounts only.

B. LT Particle Interference

Experiments similar to those described above were also done under conditions of LT particle interference. Infection in these cases was with the HR-LT stock which produces approximately 50% infective B particles and 50% defective LT particles.

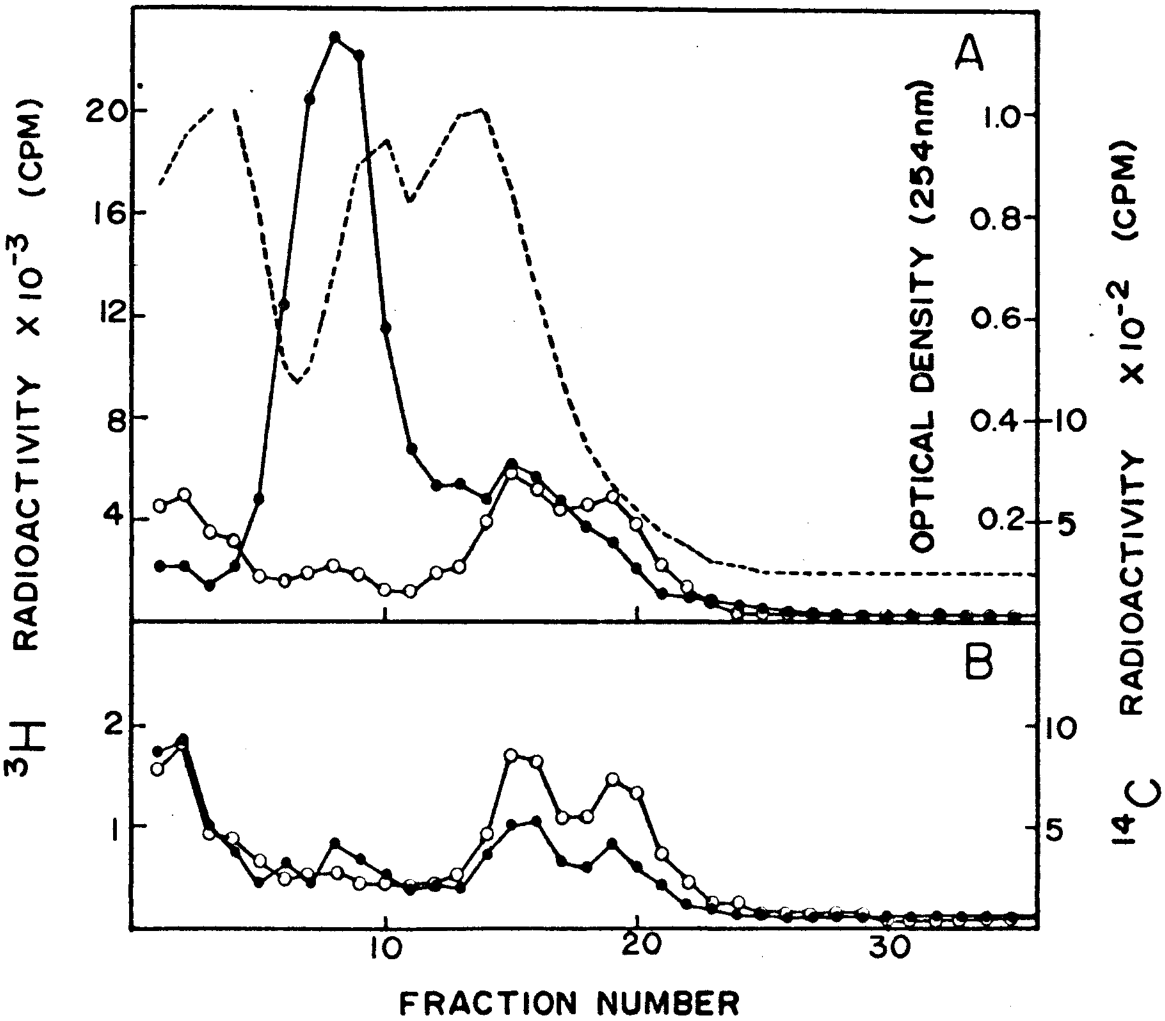
Figure 13A shows a distribution of ^{3}H-uridine and ^{14}C-amino acids obtained on a linear sucrose gradient in EDTA buffer containing a cytoplasmic extract of cells infected with the HR-LT stock. The ^{14}C-amino acid distribution shows the presence of two protein-containing structures with sedimentation coefficients of approximately 55S and 75S. The heavier protein-containing structure has a sedimentation

Figure 13. Cytoplasmic Extract of Cells Infected Under Conditions of LT Particle Interference

Cells (5 x 10^7) were exposed to ^{3}H-uridine and ^{14}C-amino acids from 3 to 6 hours post-infection with the HR-LT stock at an M.O.I. of 50 PFU/cell. The cells were harvested at 6 hours post-infection and the cytoplasmic extracts layered on a linear sucrose EDTA gradient. The gradient was centrifuged at 81,000 x g for 6 hours. The optical density (254 nm) (------) was continuously monitored as 1 ml fractions were collected. Two 0.1 ml portions of each fraction were taken and one treated with pancreatic ribonuclease (5 µg/ml) for 30 minutes at 37°C. Both portions were then acid precipitated on nitrocellulose filters and the radioactivity of ^{3}H-uridine (●—●—●—●) and ^{14}C-amino acids (○—○—○—○) monitored.

(A) before ribonuclease digestion.

(B) after ribonuclease digestion.


A
B
RADIOACTIVITY X 10^-3 (CPM)
20
16
12
8
4
2
1
3H
OPTICAL DENSITY (254nm)
1.0
0.8
0.6
0.4
0.2
RADIOACTIVITY X 10^-2 (CPM)
10
5
14C
10
20
30
FRACTION NUMBER

coefficient which appears less than that of the RNP structure observed in cells replicating only infectious virus as described earlier in this thesis. The distribution of ^{3}H-uridine shows a large amount of viral RNA at a sedimentation coefficient just less than 30S as well as in those fractions containing protein structures.

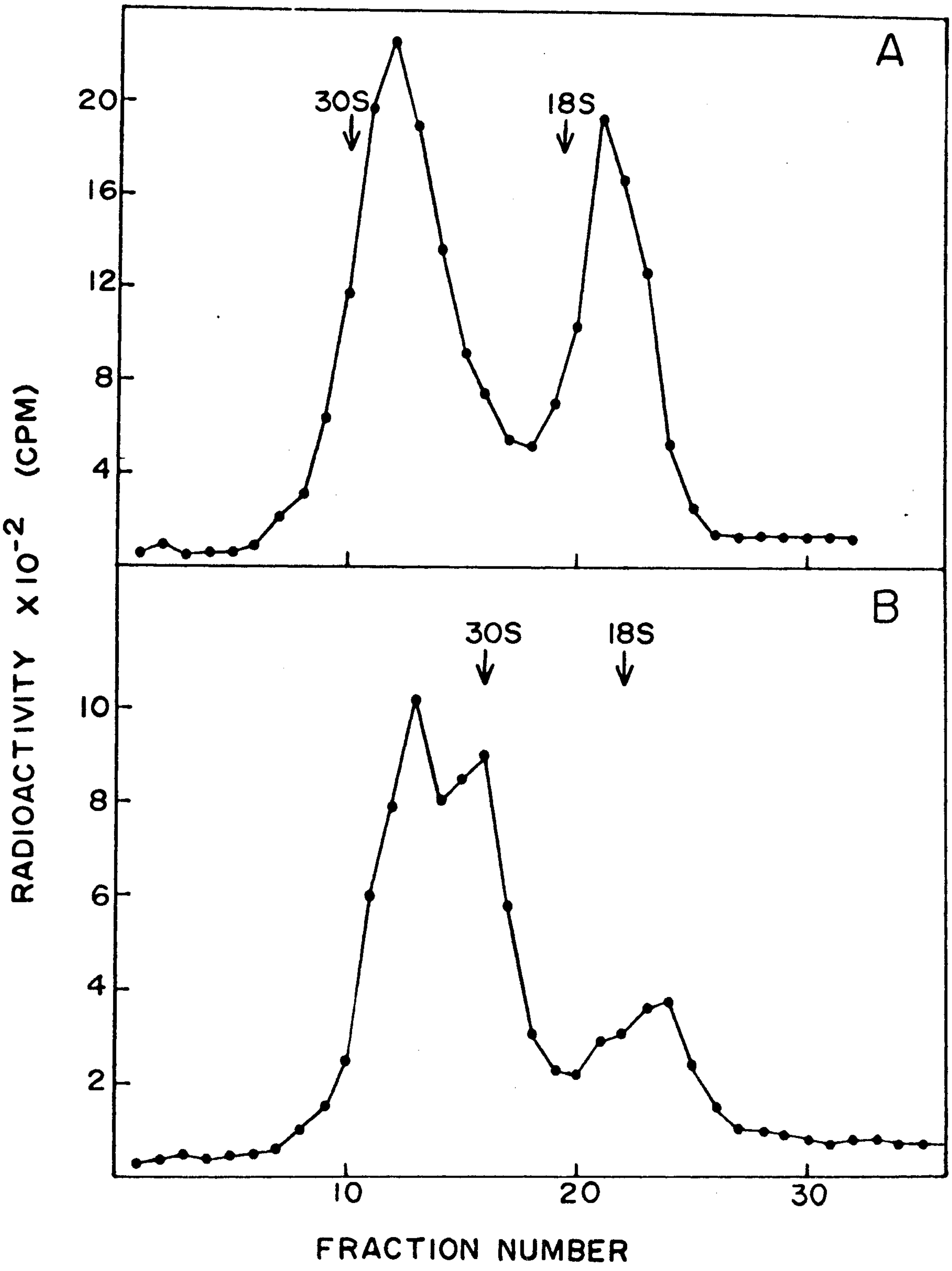
To determine whether viral RNA in the heavier fractions was present as a ribonucleoprotein complex a portion of each fraction of the gradient was treated with pancreatic ribonuclease and the acid insoluble material examined. As seen in Figure 13B the RNA associated with both of the protein peaks was partially resistant to digestion whereas the RNA sedimenting at 30S was almost totally digested. Thus both of these structures appear to be in part viral ribonucleoprotein.

As was shown in earlier experiments, 27S messenger RNA sedimented in cytoplasmic gradients at 50S and would therefore cosediment with the 55S RNP material seen in this experiment. An examination of Figures 13A and 13B shows that there is somewhat more RNA per unit protein (^{14}C cpm) associated with the 55S RNP than with the 75S RNP and further that most of this excess RNA is sensitive to ribonuclease. This result suggests there may be some 27S messenger RNA cosedimenting with the 55S RNP.

The RNA was phenol extracted from the 55S RNP region

Figure 14. RNA Extracted From the RNP of Cells Infected Under Conditions of LT Interference

Fractions 14, 15 and 16 (A) and 18, 19 and 20 (B) were pooled from the gradient shown in Figure 13 and the RNA from each set of fractions extracted. This ^{3}H-uridine labelled RNA was layered on linear sucrose gradients in STE-SDS buffer along with ^{14}C-uridine labelled ribosomal RNA marker (4000 cpm). The gradients were centrifuged at 107,000 x g for 3 hours. The gradients were collected by the dripping procedure and the radioactivity counted. ^{3}H-uridine radioactivity was plotted (●—●—●—●) and ^{14}C-uridine marker peaks were labelled with their characteristic sedimentation coefficients at 30S and 18S. The sedimentation coefficients of peaks in the distribution of ^{3}H-uridine were extrapolated from the marker values.


A
30S
18S
20
16
12
8
4
B
30S
18S
10
8
6
4
2
10
20
30
RADIOACTIVITY X 10^-2 (CPM)
FRACTION NUMBER

(fractions 14, 15 and 16) and from the 75S region (fractions 18, 19 and 20) from the gradient shown in Figure 13A. The RNA was then characterized by sucrose gradient analysis. As shown in Figure 14A, RNA extracted from the 55S region sediments with peaks at 27S and at 16S. The RNA extracted from the 75S region is shown in Figure 14B to sediment with peaks at 36S, 30S and 15S. The two species of RNA found in the 55S RNP peak are similar to the messenger RNA species found in this region under conditions of infectious virus production. Since it was shown that the 55S material contained a viral RNP complex and since it was also shown that the RNA genome of the LT particle has a sedimentation coefficent of 26S it would seem likely that the RNA peak at 27S in Figure 13A contains both messenger and LT virion RNA species. The 36S species of RNA found as a component of the 75S RNP is similar to B particle RNA seen in the larger RNP of cells infected with infectious virus alone.

DISCUSSION

A. Viral Specific Messenger RNA

Experiments performed to isolate and identify the virus specific messenger RNA species of VSV infected cells have shown two main size classes of messenger RNA, one sedimenting about 15S and another sedimenting about 27S. Both classes show over 90% complementarity to infectious B particle RNA and are similar to messenger species previously described (Huang *et al.*, 1970; Huang and Manders, 1972). The only absolute test for messenger RNA, i.e., synthesis of virus specific polypeptides from RNA in an *in vitro* system, is not yet available for routine analysis in animal virus systems. These RNA species described above do, however, fulfill some secondary criteria for messenger RNA; they are found in association with polyribosomes and dissociate from these polyribosomes in the presence of EDTA, and they fall into the correct molecular weight range to act as monocistronic messengers for the virus specific proteins. The 15S messenger species has been shown previously to separate into at least three distinct species of RNA (Petric and Prevec, 1970; Mudd and Summers, 1970). These separate species have approximately the correct molecular weights to code for protein G (M.W. = 69,000), protein

N (M.W. = 50,000) or protein NS (M.W. = 45,000) and protein M (M.W. = 29,000) (Kang and Prevec, 1969). The aggregate molecular weight of proteins N, G and M is 148,000 daltons which would require a genome with a molecular weight of 1.5×10^6. An RNA molecule of this size would have a sedimentation coefficient of approximately 26S. This fact led to the basic model of Prevec and Kang (1970), who proposed that the LT particle genome carried the coding potential for these three proteins. The aggregate molecular weight of the four small polypeptides together with protein L (M.W. = 190,000) is 383,000 daltons which accounts for virtually all of the coding potential of the 38S VSV genome (Kang and Prevec, 1971). In this last regard it is interesting to note that the 27S species of messenger RNA is of the required size to code for the 190,000 molecular weight L protein of VSV. Recent evidence of Baltimore (personal communication) lends further support to the hypothesis that the L protein is not a protein aggregate and is indeed coded for by a large messenger RNA.

Hybridization of the two classes of messenger RNA to the genome of the LT particle showed that no complementarity exists between the 27S messenger and the LT RNA while some 55% of the 15S RNA species was complementary to LT RNA. Since the LT RNA is not complementary to B particle RNA (Bishop and Roy, 1971; Galet, personal communication) these results suggest that the LT genome represents a specific segment of the B particle genome. This segment does not include that part of the genome coding for the 27S messenger and at least one of the messenger RNA molecules in the 15S size class. It does contain sequences responsible for transcription of other 15S messenger species.

The above hybridization results show that the positive 27S species in the cell does not serve as a template for synthesis of LT particle genomes nor is it a probable precursor for the 15S messenger RNA species. This latter conclusion lends further support to the hypothesis that each monocistronic messenger is transcribed independently.

B. Free Negative 27S RNA Found in Cells Producing Infectious B Particles

As seen in the RESULTS, some 30% of the 27S RNA obtained from EDTA gradients annealed in the absence of added viral RNA. This suggests that at least 15% of the isolated RNA is negative stranded. In contrast, Huang *et al.* (1970) found very little self-annealing of the 28S messenger species isolated solely from the polyribosome region of RSB° gradients. Schincariol and Howatson (1970) and Mudd and Summers (1970) found, in RNA extracted from infected cell cytoplasms, that a partially ribonuclease resistant, double-stranded RNA structure was present at sedimentation coefficients of about 30S. Virtually all of the 27S RNA described in this thesis was single-stranded after phenol extraction, and furthermore, the region of the EDTA gradient from which it was extracted did not possess significant ribonuclease resistance. The 27S RNA species did not hybridize to LT particle virion RNA. It is therefore concluded that at least 15% of the RNA in this fraction is negative stranded. It seems likely that this species is complementary to the 27S messenger which comprises 70% of the 27S RNA species.

C. The RNP Complex Formed Under Conditions of Infectious B Particle Production

Under conditions of only infective B particle production a heavy RNP complex was isolated in gradients containing cytoplasmic extracts from infected cells. These complexes consisted of RNA tightly bound with protein and are similar to subviral RNP complexes found by Petric and Prevec (1970) in the polyribosome region of RSB° gradients and by Huang _et al_. (1970), in EDTA buffer gradients. The RNA extracted from the complex was single stranded and over 70% of the RNA was similar in size to B particle virion RNA. This together with the fact that about 77% was not complementary to B particle virion RNA suggested that this RNA was identical to that of the B particle genome. Therefore the RNP complex was thought to be a subviral stage in the assembly of B particle virions. This was further supported by electron microscope examination of these molecules which showed the RNP to be helical structures similar to the RNP cores of B particle virions as described by Nakai and Howatson (1968). RNP precursors of mature virions have been demonstrated in the cytoplasm of cells infected with arbovirus such as WEE virus (Sreevalson and Allen, 1968) and Semliki Forest Virus (Friedman and Brezesky, 1967). Petric and Prevec (1970) found that the three virus proteins N, NS and L, characteristic of the VSV core were present in the RNP complexes of VSV infected cells. The virus proteins G and M which are associated with the viral envelope were not present in the complex.

About 30% of the RNA in the RNP complex had a sedimentation coefficient of between 37.5S and 30S. The self-annealing data indicated that 23% of the RNA in the complex was positive stranded. It is possible that the positive strands all arise from material sedimenting at approximately 30S. The distinct sedimentation coefficient of this RNA and the considerable separation of RNP from cytoplasmic 27S messenger RNA in EDTA gradients make it unlikely that the positive RNA represents simply contaminating 27S messenger RNA. Alternatively it is possible that the positive strands have sedimentation coefficients of 37.5S and could therefore act as templates for the synthesis of virion RNA. The definitive experiment to distinguish between these two possibilities was not performed.

D. RNP Complexes Found Under Conditions of LT and ST Defective Particle Interference

Under conditions of considerable ST particle interference an RNP complex was formed which sedimented at about 50S and contained 21S RNA similar in sedimentation coefficient to ST virion RNA. Under conditions of moderate ST interference two RNP complexes were found; a light one sedimenting at about 50S and another sedimenting at the same rate as the RNP found when only infectious particles are being produced. As above the 50S RNP complex contained RNA sedimenting at 21S similar to ST particle virion RNA. In addition, both the 15S and 27S size classes of messenger RNA were also present in this region of the gradient just as they were under conditions of only B particle production. The heavy RNP complex contained RNA sedimenting from 37.5S to 30S as was described above in B particle infected cells. In addition, some RNA with sedimentation coefficients of 21S and 15S was also found associated with this heavy RNP complex. While it is possible that these smaller RNA species resulted from contamination with material from the 50S region of the gradient, similar species were not recovered from the corresponding RNP observed in B particle infected cells.

Similar observations were obtained under conditions of LT particle interference. The light RNP complex sedimenting at 55S found under these conditions contains RNA which sediments at 27S and 15S similar to the messenger species. The 55S RNP complex was partially ribonuclease resistant. This nuclease resistance was not found in the 50S peak containing messenger RNA under conditions of only B particle production. It is quite likely that the 55S RNP complex contains the 26S RNA species characteristic of the LT virion RNA. In this experiment the 26S RNA would be hidden in the large peak of 27S messenger. The heavy RNP complex produced under LT interference contained significant amounts of RNA sedimenting at 36S similar to B particle virion RNA, at 36 to 30S, and at 15S similar to messenger RNA.

The above results show, in agreement with previous work by Petric and Prevec (1970), and Wild (1971), that besides the presence of RNP complexes characteristic of replicating B particles, cells co-infected with defective particles also contain smaller RNP complexes characteristic of the defective particles. Three RNP complexes with sedimentation coefficients of 140S, 110S and 80S containing RNA species similar to B, LT and ST virions respectively were recently reported by Kiley and Wagner (1972). These three RNP complexes contained the characteristic core proteins N, L and NS. The protein NS could be readily dissociated from the complex in high salt. As infected cells which produced these three cytoplasmic RNP complexes released B, LT and ST virions, it was therefore postulated that the RNP complexes were precursors to these virions.

In contrast to the RNP complexes which can be isolated from purified virions by detergent treatment, it can be seen from work in this thesis that RNP complexes from the infected cell contain not only the RNA species characteristic of the particle but appear as well to have other associated RNA species. In particular the heavy complex which is less likely to be contaminated with free messenger RNA than the lighter complexes, still shows a heterogeneity of RNA size classes from 30 to 37.5S. This result may indicate that the RNP complexes are active in the cell either as transcriptive or as replicative structures. It has been shown in our laboratory (Galet, Shedlarski and Prevec, 1972) that the RNP complexes in B particle infected cells possess in vitro transcriptase activity.

At least two hypotheses have been put forth regarding the mechanism of T particle mediated interference. The first of these suggests that a product (perhaps virus envelope protein) of the genetic information of the defective particle inhibits the transcription from the B particle genome of a messenger coding for virus replicase. Under this condition it might be expected that both replication and consequently messenger RNA transcription would be reduced during interference. A second model suggests that the defective particle genome behaves as a competitor with B particle genomes for the available replicase enzyme. Due to their shorter length and presumably correspondingly short replication time the defective particles could very quickly gain a selective advantage within the cell. The results in this thesis tend to be in general agreement with this latter model. Under conditions of moderate interference by either LT or ST defective particles there was considerably more of the RNP complex characteristic of LT and ST particles replicated relative to the RNP characteristic of B particle virion. All of the messenger RNA species transcribed under conditions of only B particle production were, however, still present in considerable amounts. Secondly, during very severe interference with ST particles, under conditions in which relatively little messenger RNA or RNP characteristic of B particles was present in the cell, there was nevertheless a large amount of RNP characteristic of ST particles. This result suggests that ST RNP replication had a very large competitive advantage over the B particle RNP in this situation.

Grimely *et al*. (1972) and Friedman *et al*. (1972) have recently reported that the RNP complexes which are precursors to virions of Semliki Forest Virus, an arbovirus, may be associated with particular membrane structures formed in arbovirus infected cells. Picorna virus have also been shown to replicate in association with cytoplasmic membrane structures (Caliguiri and Tamm, 1970).

In this regard absence of any positive indication for replication complexes from this work or work done *in vitro* may suggest that the membrane associated structures, which are removed with the nuclei in preparing cytoplasmic extracts, should be carefully re-examined for possible intermediates in virus replication.

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