THE GENERATION OF DEFECTIVE INTERFERING RUBELLA VIRUS PARTICLES

by

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Abstract

Rubella virus (RV) has been propogated in murine (L-2) fibroblasts and viral titers determined using a modified hemadsorption assay. The titration results suggested that rubella virus populations may be comprised of standard and defective virions.

RV stocks were examined for their ability to interfere with standard (low interference) rubella virus. High interference RV stocks could reduce standard RV infectivity by greater than 99%.

A new purification procedure has been developed in order to examine the virions produced by high and low interference RV stocks. Rubella viral particles have been purified using a discontinuous renografin gradient followed by a continuous renografin gradient. These gradients allowed separation of intact purified virions from contaminating host membranous material. Greater than 90% of the total original infectivity was recovered as determined by the hemadsorption assay. Electron microscopic examination demonstrated the presence of intact rubella virions.

Virions from high and low interference RV stocks have been purified, the RNA extracted from the purified virions, labeled with ^{125}I, electrophoresed on 5% polyacrylamide gels

and subjected to autoradiography. Virions from low interference RV stocks were contained in one band at a density ρ = 1.19 gm/cc^3. The two single-stranded RNA molecules that could be extracted from these virions have molecular weights of 2.95 and 2.80 x 10^6 daltons. Virions purified from high interference RV stocks were contained in at least three bands at densities ρ = 1.19, 1.17 and 1.15 gm/cc^3. RNA molecules with molecular weights of 2.95 and 2.80 x 10^6 daltons could be extracted from the virions banding at a density ρ = 1.19 gm/cc^3. Virions banding at the lighter densities ρ = 1.17 and 1.15 gm/cc^3 contained RNA molecules of 1.25 and 1.05 x 10^6 daltons in molecular weight respectively.

A fresh isolate of RV has been serially passaged in L-2 cells in order to observe the sequential generation of defective viral particles. The virions from each passage have been purified, the RNA isolated, labeled and analyzed by PAGE. The molecular weights of the RNA molecules isolated indicated the possible cyclic generation of defective viral particles.

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Abbreviations used in this thesis are:

RV:	Rubella virus
SV:	Sindbis virus
SFV:	Semliki Forest virus
L-2:	Murine fibroblasts
MEM:	Minimal essential medium
FCS:	Fetal calf serum
TNE:	0.15m NaCl, 50mM Tris-HCI and 1mM EDTA, pH 7.8
TNM:	0.15m NaCl, 50mM Tris-HCI, 1mM MgCl , pH 7.8
TCA:	Trichloroacetic acid
ELISA:	Enzyme linked immunoadsorbant assay
HAd:	Hemadsorption
M.o.i:	Multiplicity of infection
SDS:	Sodium dodecyl sulfate
BSA:	Bovine serum albumin
PAGE:	Polyacrylamide gel electrophoresis
HA:	Hemagglutination protein
BHK-21	Baby hamster fibroblasts
NDV:	Newcastle disease virus
O/N:	Over night
D.I.	Defective interfering
Srbc.:	Sheep red blood cells
PBS:	Phosphate buffered saline

ACKNOWLEDGMENTS

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Introduction

Defective interfering viral particles

Defective interfering (D.I.) viral particles as defined by Huang have the following properties: (12,14) (1) they contain a part of the standard viral genome and as such they can be considered viral deletion mutants. (2) they contain the same viral structural proteins as the standard virus. (3) because they are viral deletion mutants they are unable to replicate on their own but can replicate in the presence of standard virus. (4) although they require standard virus to replicate, at the same time they inhibit standard virion replication and are therefore interfering.

The effects of D.I. particles were first observed on serial undiluted passaging of influenza virus in embryonated eggs.(45) A cyclic decrease in the ratio of infectivity to hemagglutination was observed and this observation is now known as the von Magnus phenomenon. (Figure 1) It was further demonstrated that a serially passaged influenza preparation of low infectivity could inhibit the growth of an influenza preparation of high infectivity. The viral particles from low infectivity stocks contained less nucleoprotein per virion than virions isolated from standard preparations with high infectivity (23).

Since this initial observation of D.I. particle

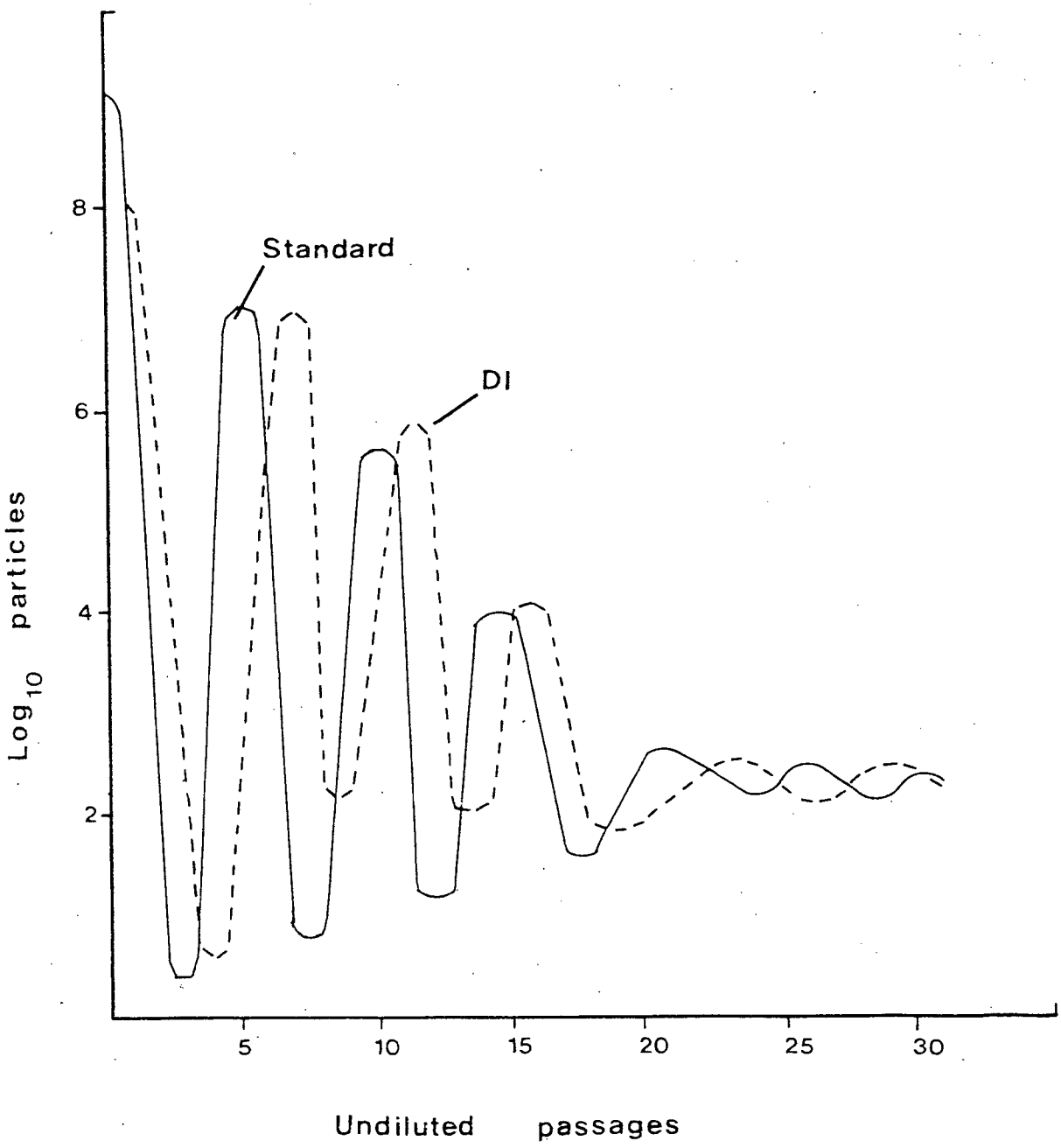

Standard
DI
8
6
4
2
Log10 particles
5
10
15
20
25
30
Undiluted passages

Figure 1

generation by influenza virus the presence of D.I. particles has been demonstrated in almost every animal virus system studied. In most cases it is difficult to obtain pure preparations of D.I. particles uncontaminated by standard virions. This is due primarily to the small differences in the virion densities. Under these circumstances not only is it difficult to demonstrate the inability of the defective virions to replicate on their own but it is also impossible to establish unequivocally that the interference characteristics of a given stock are due to the defective virions. However the lower infectivity of a defective containing stock as compared to a stock free of defectives is considered to be consistent with the presence of D.I. particles.

Rubella virus

Rubella virus (RV) is a member of the family Togaviridae. RV is noncytopathic and replicates in the host cell cytoplasm. The virions mature on budding through the cytoplasmic membrane and are therefore enveloped. Difficulties have been continually encountered in attempts to propogate and characterize the virus. A variety of procedures have been reported for rubella purification from infected tissue culture media. These procedures include (a) concentration of the virus using ammonium sulfate

precipitation (1) (b) dialysis against polyethylene glycol (PEG)(4) and (c) centrifugation on a discontinuous sucrose gradient followed by sedimentation through a continuous sucrose gradient (22).

Although there have been numerous reports of rubella purification there are no consistent data concerning the recovery of virus infectivity, the purity of the virus preparations or the number of structural proteins present in the intact virion. The problems encountered in purifying the virus are further exacerbated by the low and variable yields of virus. Varying densities (ρ = 1.16 - 1.23 gm/cc^{3}) have been reported for rubella virions (26, 34, 37, 41). Perhaps these discrepancies could be accounted for by a mixed population of standard and defective particles.

The rubella genome consists of a single-stranded infectious RNA sedimenting at 40S in 0.1 M saline at 20°C. The molecular weight based on these data was calculated to be 3.0 x 10^{6} daltons (11). RNA isolated from partially purified virions and fractionated by rate zonal centrifugation in sucrose density gradients indicated the bulk of RNA sedimented as a sharp band of 38S (35). In addition, however, a 25S RNA'se sensitive component was always detected. The authors have suggested that this 25S RNA could represent the genome of incomplete defective virus particles. There has been an additional report which suggests the existence of D.I. Rubella particles (27).

Sindbis virus and Semliki Forest virus both togaviruses, have been studied in greater depth than Rubella and have been found to generate defectives with relative ease (39). Although there has been no direct report of the presence of Rubella D.I. particles, information in the literature indicates that Rubella should be able to generate D.I. particles.

The objective of this thesis was to determine if rubella virus could generate defective-interfering viral particles.

Materials and Methods

Cells and Media

Murine fibroblasts (L-2) cells were a kind gift from W. Flintoff (Department of Bacteriology and Immunology, University of Western Ontario). The cells were routinely propogated as monolayers at 37°C in a humid atmosphere containing 5% CO_2 with Minimal Essential Medium (MEM) supplemented with 10% fetal calf serum (FCS; Grand Island Biological Company, GIBCO), 100 µg/ml streptomycin and 100 I.U./ml penicillin.

Virus

Rubella virus (RV), strain 2872R, was obtained from G.D.M Kettyls (British Columbia Public Health Laboratories, Vancouver, British Columbia) as the first passage after primary isolation. Stock virus was routinely prepared by inoculating semiconfluent monolayers of L cells with RV at a multiplicity of infection (m.o.i.) of 0.01. After adsorption at 34°C in a humid atmosphere containing 5% CO_2 for 1 1/2 hr, additional medium was added and the flask was incubated at 34°C for 6 days, at which time the culture supernatant was collected and frozen at -80°C.

The hemadsorption assay

Serial doubling dilutions of RV suspensions were used to infect confluent monolayers of L-2 cells grown in tissue culture chamber slides (Lab Tek Products, Division of Miles Laboratories, Inc., Naperville, Illinois 60540). Two-chamber slides were used. Each chamber received a 50 µl aliquot of the appropriate RV dilution. Virus was allowed to adsorb for 1 hr at 34°C and 2.5 ml of medium and 50 µl of a 20% suspension of heparinized sheep erythrocytes in Alsever's solution were added directly to each chamber. The slides were then incubated for 24 hours at 34° C. The plastic chambers were removed and each slide was washed gently by immersion in pH 7.4 Dulbecco phosphate-buffered saline (1954) at room temperature and examined microscopically for hemadsorbing cells. Uninfected control monolayers were treated in an identical fashion.

Those samples pretreated with rubella specific antiserum (H.I. titer 1:128) were incubated with an equal volume of a 1/32 dilution of the antiserum. The samples were incubated for 1 hr. at 34° C prior to adsorption on the L-2 monolayers. These samples were then treated in an identical manner to the infected monolayers. In mixing experiments an equal volume of a high interference stock was mixed with a low interference stock. These samples were then treated in an identical fashion to the rubella samples.

Purification of rubella

L cell monolayers were infected at an m.o.i. of 0.01 and incubated at 34°C for 6 days in MEM containing FCS, penicillin, streptomycin and ^{3}H-uridine as described. The culture supernatants were collected and centrifuged at 3000 x g for 20 minutes. All procedures were carried out at 4°C unless otherwise stated. The supernatant obtained was recentrifuged at 100,000 x g for 3 hours and the resulting pellet was resuspended in 0.2 ml TNE buffer (0.15M NaCl, 50 mM Tris-HCl and 1 mM EDTA, pH 7.8). This sample was layered onto a 16 ml 25-45% (wt/vol) discontinuous Renografin-60 (Diatrisoate Meglumine, Squibb) gradient prepared with TNE buffer and centrifuged in an SW 27 rotor at 55,000 x g for 2 hr. The single, sharp band at the interface was collected, pelleted as described previously, resuspended in 0.5 ml TNM buffer (0.15 M NaCl, 50 mM Tris-HCl, 1mM MgCl , pH 7.8) and layered on a 12 ml 30-45% (wt/vol) continuous Renografin gradient prepared with TNM buffer. After centrifugation at 200,000 x g for 3 hr, 0.5 ml fractions were collected. A 25 µl aliquot from each fraction was precipitated with trichloroacetic acid (TCA). The precipitates were retained on glass-fiber paper discs (Reeve Angel, N.J.), washed three times with cold, 5% TCA, followed by 95% ethanol, dried and counted. A second aliquot was removed from each fraction for an enzyme-linked immunoadsorbent assay (ELISA). Appropriate

fractions were then pooled, diluted with TNM buffer and centrifuged at 100,000 x g for 3 hr to remove the Renografin.

ELISA - Enzyme-linked Immunoadsorbent Assay

The ELISA was performed according to the procedure described by Voller (44). A 50 µl sample was removed from each fraction obtained from the continuous gradient. It was diluted 1/10 in coating buffer. Duplicate 200 µl aliquots of the filtered sample were then adsorbed to wells of a microtiter plate (Cooke Laboratory Products, Dynatech Laboratories Inc., 900 Slaters Lane, Alexandria, Va. 22314). After coating, a predetermined 1/16 dilution of human antirubella antiserum (HI titer = 1/128) was added to each well. Antibody binding was measured using a previously determined 1/2000 dilution of rabbit antihuman IgG (Flow Laboratories) linked to alkaline phosphatase. The $A_{400\,m\mu}$ was determined after 30 min incubation at room temperature. Renografin had no effect on ELISA activity.

Electron Microscopy on Gradient Fractions

Gradient fractions were negatively stained by mixing the sample with an equal volume of 3% phosphotungstic acid, pH 7.0. The samples were placed on Formvar-carbon coated grids and excess fluid was withdrawn. The grids were examined in a

Philips EM 301 electron microscope.

Radiolabeling of Rubella RNA

Virus to be labeled was grown as previously described. After adsorption for 1 1/2 hr. at 34°C, MEM containing 10% FCS, 100 µg/ml streptomycin, 100 I.U./ml penicillin and 3 µCi/ml ^{3}H-uridine (New England Nuclear, Boston, Massachusetts, U.S.A.) was added. Virus was harvested 6 days post infection. The radiolabeling aided in detection of rubella after isopycnic gradient centrifugation. However the low specific activity of the ^{3}H -label and the poor virus yields made it necessary to iodinate the RNA prior to further analysis.

Isolation of Rubella RNA

The isolation of viral RNA has been described (35). Following isopycnic gradient centrifugation appropriate peak fractions were pooled, diluted with TNM buffer and centrifuged at 100,000 x g for 3 hours. The RNA was released from the purified virions by treatment with TNE buffer containing 1% SDS at room temperature. The released viral RNA was deproteinized by phenol treatment (22°C/10 min.). The aqueous phase was removed and the NaCl concentration adjusted to 1.0M. The nucleic acid was precipitated by 3 volumes of ethanol at -20°C/O.N. The precipitate was

pelleted, dried and was then ready for iodination.

Iodination of Rubella RNA

The Prensky modification (32) of the Cummerford reaction (6) was used for the iodination of rubella RNA as follows:

To 5 µl RNA solution

5 µl H_2O

2 µl buffered thallic ion solution

was added.

5 µl Na^{125} iodide (Amersham)

10 µl acidification reagent.

The reaction mixture was incubated in a water bath at 60°C/15 minutes. The pH monitored to pH 4.7. After 15 minutes incubation 0.5 ml TNE solution containing 0.001M Na_2So_3 was added and the RNA incubated an additional 20-30 minutes at 60°C. The sample was then cooled and iodinated RNA separated from unreacted ^{125}I by G-25 sephadex column chromatography on a 25 cm x 1 cm column. The RNA was eluted using ammonium acetate 0.1 M pH 5.0 buffer containing 0.2% SDS.

All reaction mixtures were either pretreated with bentonite (8) or contained 0.2% SDS to inactivate any contaminating RNA'se. All glassware had been sterilized for 30 minutes and gloves were worn during manual manipulations.

Eluted 1 ml fractions were collected following G-25 Sephadex column chromatography. A 10 µl aliquot was removed,

diluted 100 X and counted. Fractions containing the iodinated RNA were pooled and the RNA precipitated by 3 volumes of ethanol at -20°C/ON in the presence of 100 µg cold carrier yeast RNA. The dried precipitate was resuspended in sample buffer. A small aliquot was removed to determine RNA'se A sensitivity (Sigma Chemicals). After one hour incubation at 37°C in the presence of 10 µg/ml RNA'se A TCA precipitable counts were determined in the presence of cold carrier BSA. In all cases less than 15% of original counts were preciptable following RNA'se A digestion.

PAGE - Polyacrylamide slab gel electrophoresis

^{125}I-labeled RNA samples were resuspended in TBE sample buffer (45mM Tris, 40mM boric acid and 1.24mM EDTA). The samples were applied to a 10 cm. slab gel (5% acrylamide and 6 M urea made up in TBE). The RNA was electrophoresed for 3 hours at 25 ma. The gel was then fixed for 1 hr. at 22°C in 25% isopropanol and 10% acetic acid. The gel was dried onto filter paper backing and subjected to autoradiography using Kodak NS-2T film. Exposure time depended on the number of counts applied to the gel. The time would vary from 3-14 days exposure.

Calibration of the PAGE system

The acrylamide gels were calibrated using three

molecular weight markers. RNA was extracted from Sindbis virus (a kind gift from Pierre Talbot, Department of Biochemistry, U.B.C.) in an identical manner to rubella. In 6 M urea Sindbis RNA has a M.W. of 2.1×10^6 daltons. E. coli ribosomes were prepared and the 30S (1.0×10^6 daltons) and 50S (1.67×10^6 daltons) ribosomal RNA isolated (18). Sindbis RNA and E. coli rRNA were iodinated as rubella RNA. These iodinated markers were then electrophoresed with rubella RNA in order to calibrate the gel.

Containment

All manipulations involving rubella were conducted in a certified B level viral containment hood.

Iodination reactions were carried out in a fume hood protected with lead shields.

Results

I The hemadsorption assay

Rubella (RV) is a noncytopathic virus and direct cytopathic assays are not effective in titering the virus. Quantitation has been traditionally based on microfoci formation (7, 40) or the interference principle (25, 28). The interference assay is lengthy and tedious. Prior infection with rubella renders a cell immune to superinfection with Newcastle Disease Virus (NDV). Cells uninfected with rubella succumb to the cytopathic effects of NDV. The rubella infected cells are allowed to grow into colonies which are then enumerated to obtain rubella titers. The development of a rapid and accurate quantitation technique became desireable.

The hemagglutination (HA) protein in rubella infected BHK-21 cells has been reported (10, 38). The expression of the HA protein on the cell surface allows erythrocytes to adsorb to the surfaces of rubella infected cells. A hemadsorption assay has been developed which enables non-cytopathic mutants of NDV to be detected (24). A modification of this assay was developed in order to detect non-cytopathic wild type RV microfoci formed in rubella infected L-2 monolayers.

The virus infected monolayers were grown in the presence

of sheep erythrocytes and 24 hours post infection the hemadsorbing foci were enumerated. These hemadsorbing foci were easy to score and titers of infectious rubella virus could be obtained (Figure 2). After infectious units were scored they were plotted as a function of concentration and compared to expected theoretical results (2) (Figure 3).

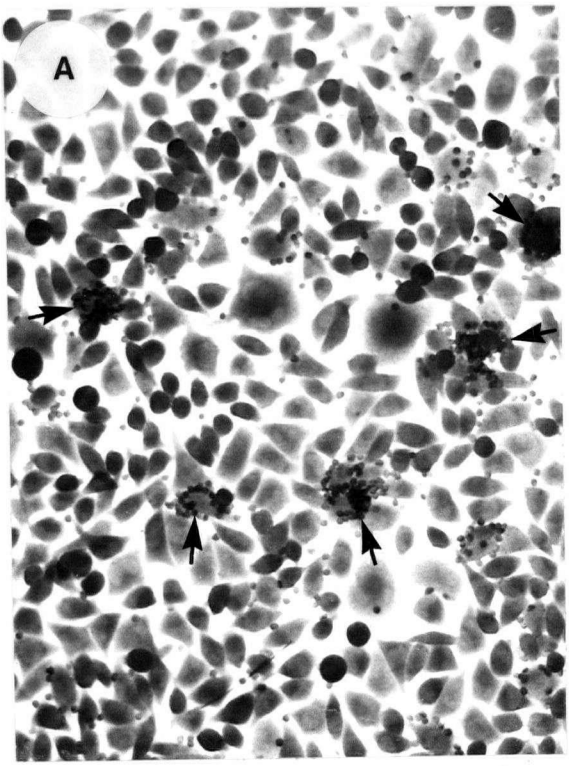
The theoretical expected results are expressed as either one-hit or two-hit kinetics. One-hit kinetics occurs when the decrease in infectious units is proportional to the 1st power of the virus concentration. One virion is able to initiate an infection. Two-hit kinetics results when the decrease in infectious units is proportional to the 2nd power of the concentration. Two populations of virions are present and both are required to initiate a successful infection. A cooperative effect is operative.

When rubella infectious units were plotted as a function of concentration the decrease in HAd units was proportional to the fractional power of the concentration. These results suggested that two populations of virions may be present in the virus stock and an interference effect might be operative. Viral stocks were then examined for their interference characteristics.

Figure 2A - Quantitation of Rubella virus using the hemadsorption assay.

Cells were infected with serial doubling dilutions of rubella and incubated in the presence of srbc for 24 hours at 31°C. The hemadsorbing foci were visible after removal of the medium and three washes with PBS. Arrows indicate hemadsorbing foci.

Figure 2B - Control cells were incubated with medium and treated in an identical manner as the infected cells. Only residual single srbc's remained after washing with PBS.


A

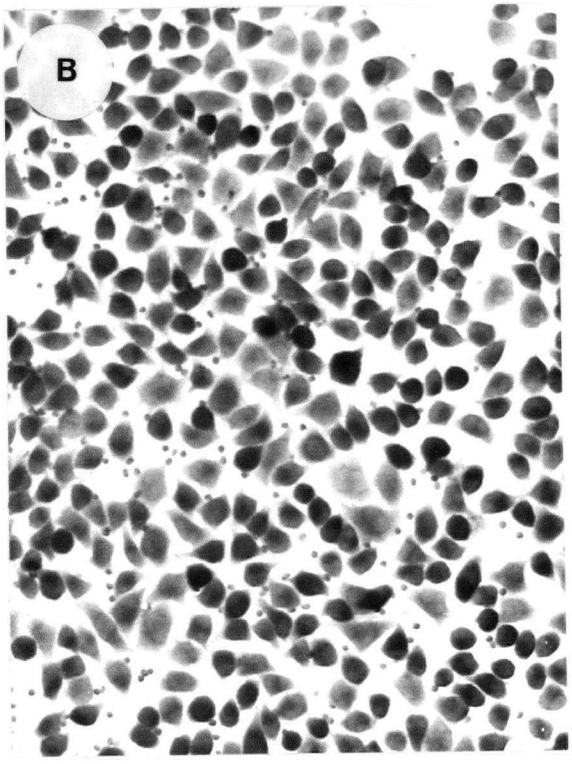

B

Figure 3. Titration of rubella virus using the hemadsorption assay.

■——■ rubella HAd infectious units plotted as a function of concentration.

▲— —▲ theoretical expected results when the decline in the number of infectious units is proportional to the first power of the virus concentration. (1 hit kinetics).

Δ----Δ theoretical expected results when the decline in the number of infectious units is proportional to the second power of the virus concentration. (2 hit kinetics).

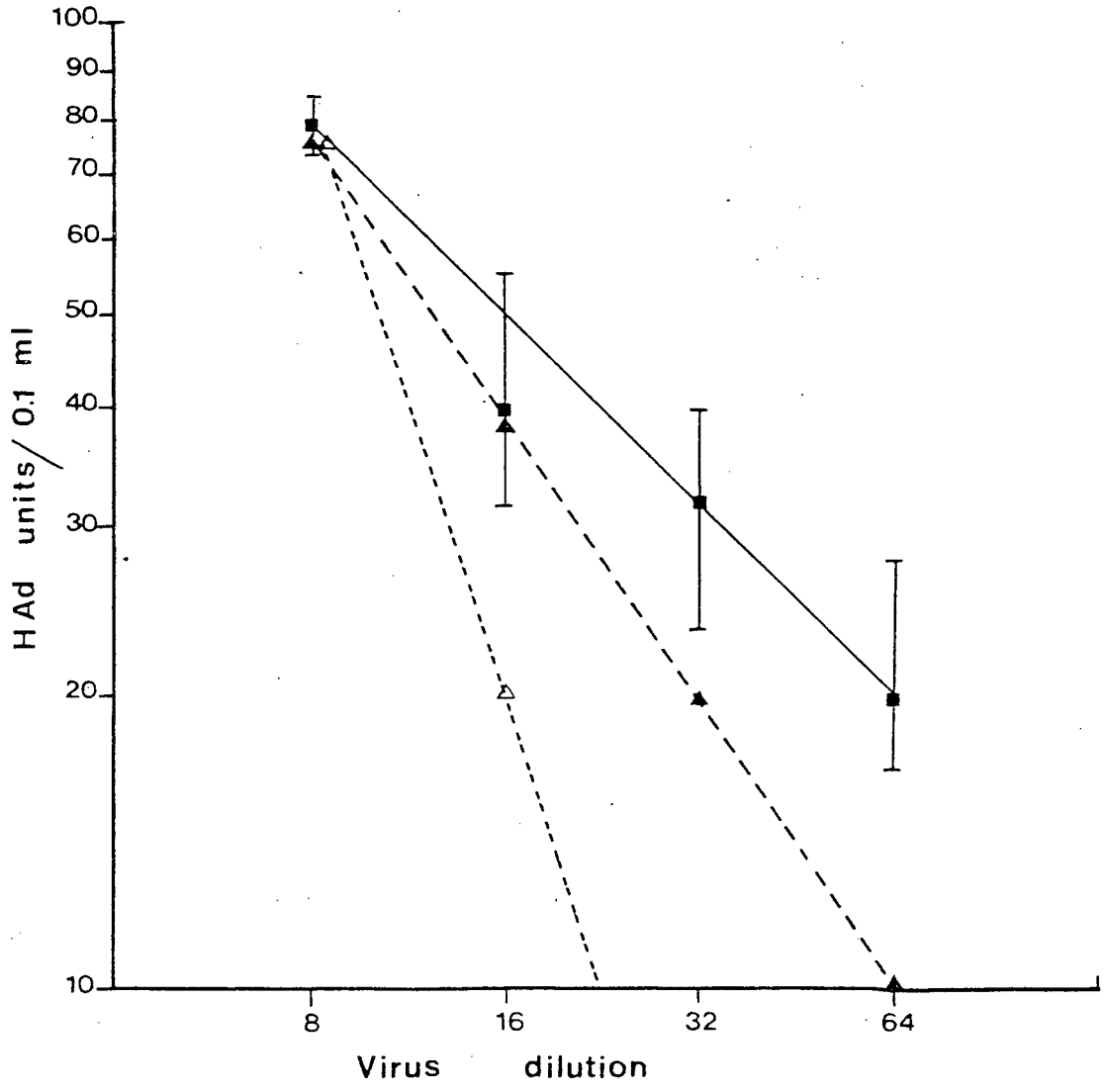

100
90
80
70
60
50
40
30
20
10
HAd units/0.1 ml
8
16
32
64
Virus dilution

Figure 3

II Rubella interference assay

Viral stocks were titered and infectious units plotted as a function of concentration. Stocks which demonstrated high degrees of autointerference were mixed with low autointerference stocks. Interference was measured as decrease in infectious units as a function of concentration. (Figure 4). When the high interference stock (4.48×10^4 HAd units/ml) was mixed with the low interference stock (5.12×10^4 HAd units/ml) infectivity was reduced to 1.60×10^2 HAd units/ml. Pretreatment of all samples with specific anti-rubella serum reduced the number of infectious centers to background levels.

When the high interference stock was mixed with the low interference stock there was 99.7% inhibition of virus infectivity (Table I). These results further suggested that two populations of virions, both standard and defective, might be present in the high interference stock. Virions from both high and low interference stocks were purified in an attempt to isolate the defective particles.

III Purification of Rubella virus

Discontinuous and continuous renografin gradients were utilized in order to separate intact virions from contaminating host membranous material. The virions and the

Figure 4. Titration of low and high interference rubella stocks.

■——■ Titration of a high interference RV stock.
□——□ pretreatment of high interference RV stock with specific anti-rubella serum

▲— —▲ Titration of a low interference RV stock.
△— —△ Pretreatment of low interference RV stock with specific anti-rubella serum.

●— —● Titration of low interference RV stock mixed with an equal volume of the high interference RV stock.
○— —○ Pretreatment of the mixed low and high interference stocks with specific anti-rubella serum.

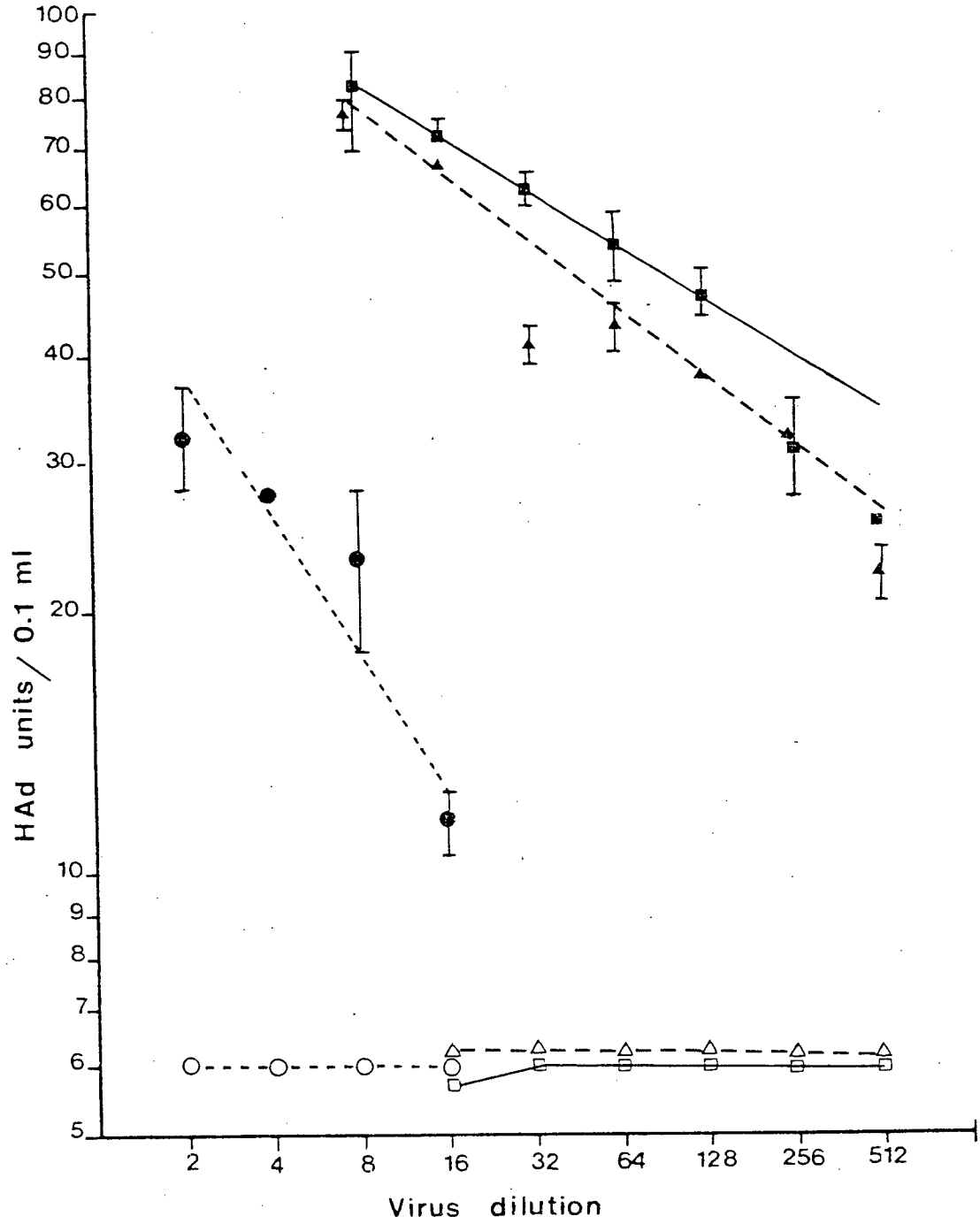

100
90
80
70
60
50
40
30
20
10
9
8
7
6
5
HAd units / 0.1 ml
2
4
8
16
32
64
128
256
512
Virus dilution

Figure 4

TABLE I

RUBELLA VIRUS INTERFERENCE

VIRUS STOCK	HAd UNITS/ml	%INHIBITION
Low interference	5.12×10^4	
High interference	4.48×10^4	
Low & high interference	1.6×10^2	99.7

host contaminants have different densities and they could be easily separated on the renografin gradients. The purification procedure outlined in Figure 5 was followed.

Fractions were collected following isopycnic gradient centrifugation. An aliquot was removed to measure ^{3}H-uridine incorporation into TCA precipitable counts (Figure 6). Additional aliquots determined ELISA activity (Figure 7A) and HAd infectivity (Figure 7B). Purification by isopycnic gradient centrifugation indicated 3H-uridine incorporation into two peaks. ELISA activity detected rubella antigens in these two peaks. The HAd assay demonstrated that 90% of original virus infectivity was located in the centre peak and less than 2% was located in the light density peak.

Electron microscopic examination demonstrated the presence of intact virions in the centre peak. (Figure 8). Sindbis virus which has a similar density (ρ= 1.196 gm/cc^{3}) to rubella virus cosedimented with this peak. Virus recovery was monitored at each step during purification.

Greater than 90% of original infectivity was recovered (Table II). Polyacrylamide gel analysis of ^{125}I-labeled viral proteins indicated that high molecular weight contaminants present in sucrose gradient purified virions were eliminated from viral preparations purified on renografin gradients. The virus preparation was considered pure as there was no further loss of polypeptides with retention of 90% of

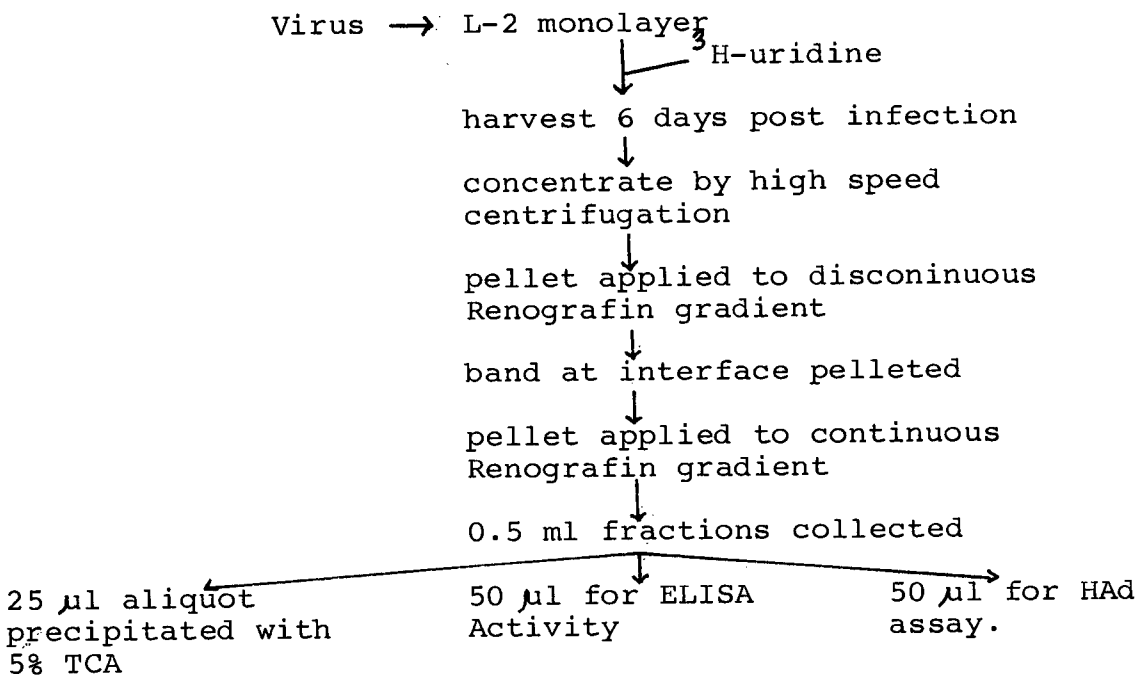
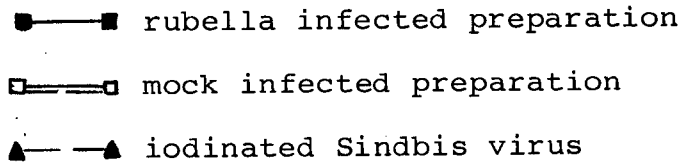

Virus
L-2 monolayer
^{3}H-uridine
harvest 6 days post infection
concentrate by high speed centrifugation
pellet applied to disconinuous Renografin gradient
band at interface pelleted
pellet applied to continuous Renografin gradient
0.5 ml fractions collected
25 µl aliquot precipitated with 5% TCA
50 µl for ELISA Activity
50 µl for HAd assay.

Figure 5

Figure 6 - Isopycnic gradient centrifugation analysis of Rubella virus.



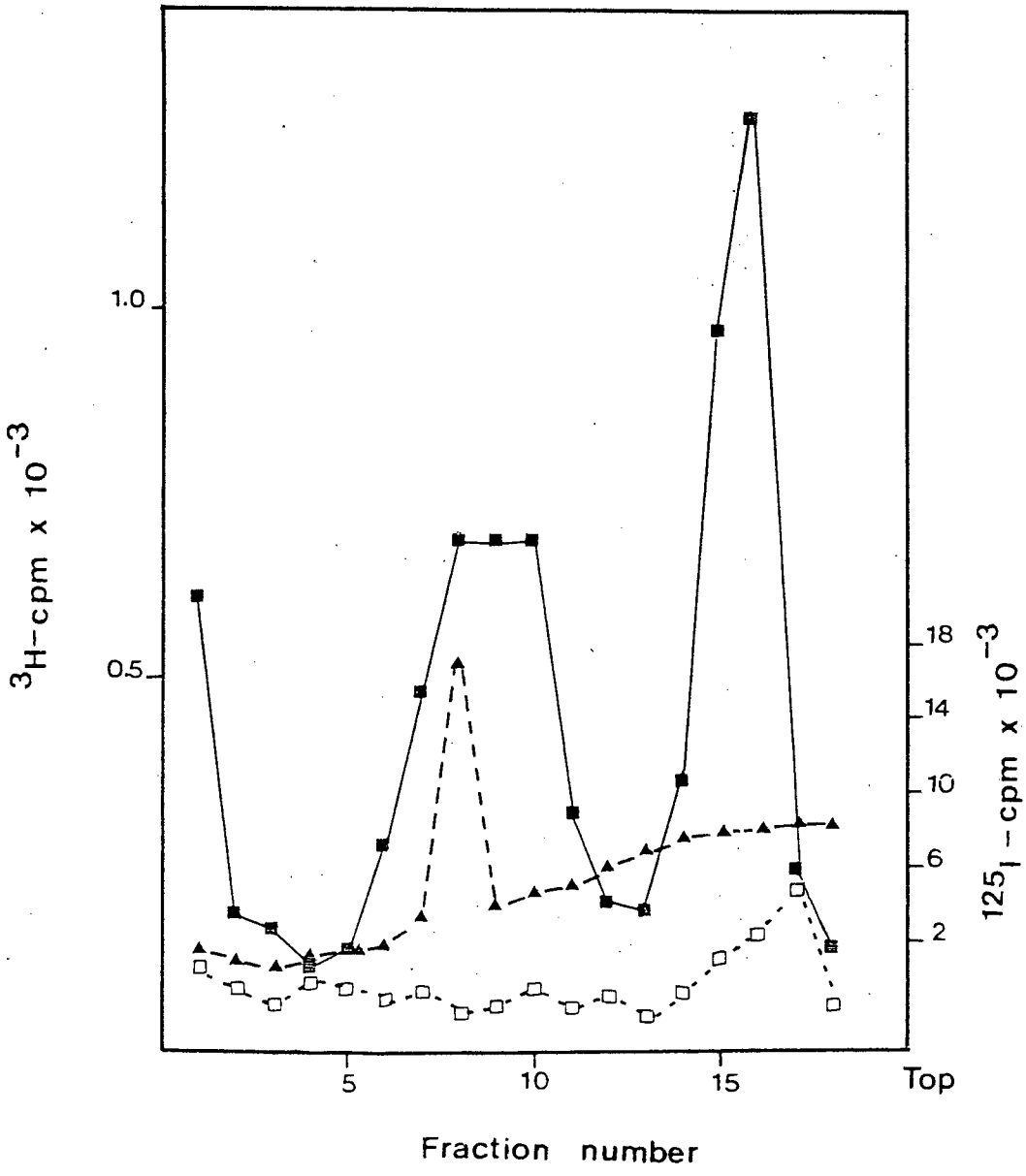

1.0
0.5
^{3}H-cpm x 10^{-3}
18
14
10
6
2
^{125}I-cpm x 10^{-3}
5
10
15
Top
Fraction number

Figure 6

Figure 7A - ELISA activity of rubella following isopycnic gradient centrifugation.

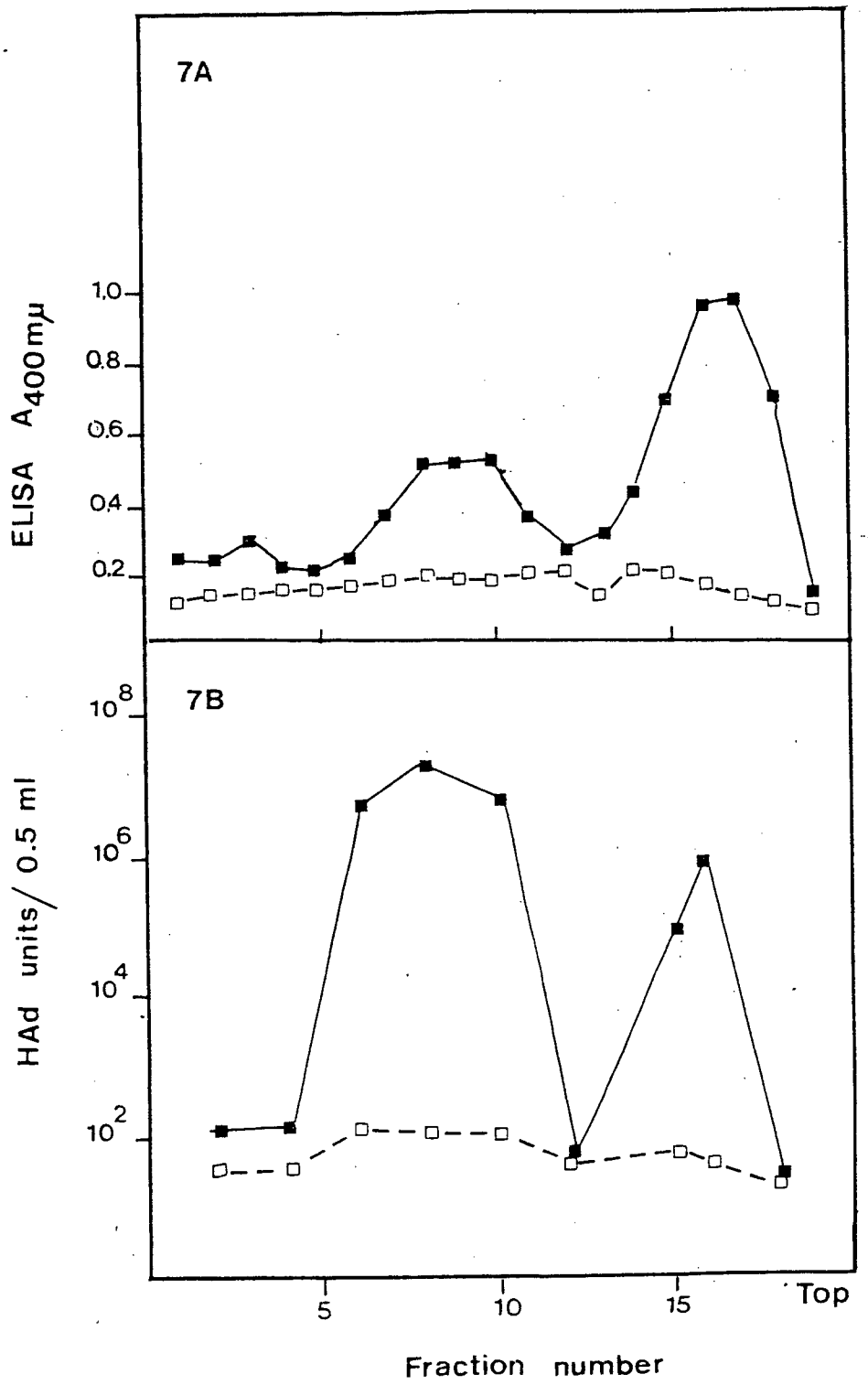
■——■ rubella infected preparation

□— —□ mock infected preparation

Figure 7B - HAd activity of rubella following isopycnic gradient centrifugation.

■——■ rubella infected preparation

□— —□ mock infected preparation


7A
ELISA $A_{400m\mu}$
1.0
0.8
0.6
0.4
0.2
7B
HAd units/ 0.5 ml
10^8
10^6
10^4
10^2
5
10
15
Top
Fraction number

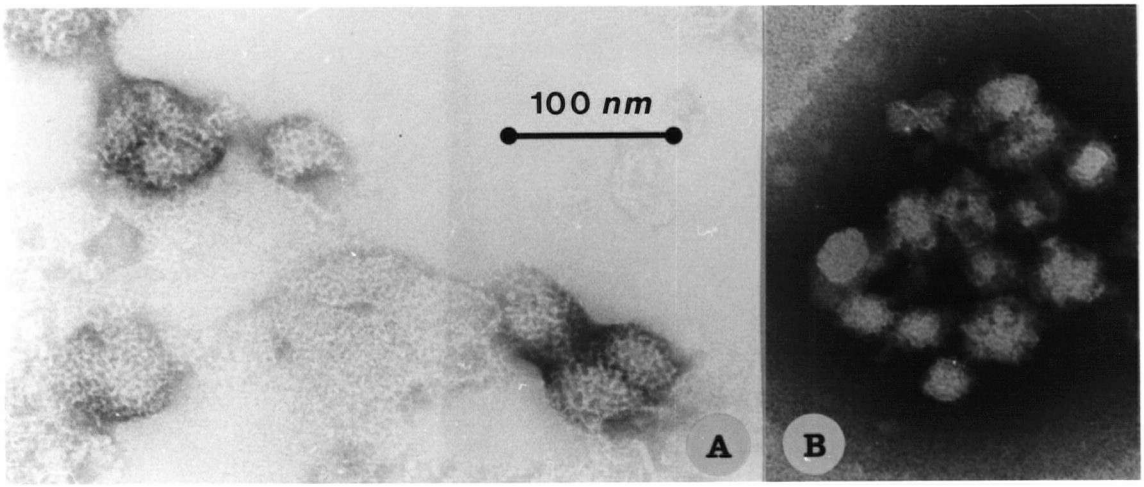


Figure 8A - Electron micrograph of rubella virions contained in the centre peak following isopycnic gradient centrifugation.

Figure 8B - Rubella cores following Triton X-100 treatment of rubella virions.

TABLE II

Recovery of Rubella during purification

VIRUS PREPARATION	HAd Units/ml	Total HAd Units	% Recovery
infected tissue culture medium	5.80×10^5	6.61×10^7	100
sample applied to discontinuous gradient	3.96×10^7	6.34×10^7	95.8
band removed from discontinuous gradient	3.27×10^7	6.40×10^7	96.8
sample applied to continuous gradient	6.90×10^7	6.26×10^7	94.7
fractions 14-16 from continuous gradient	9.80×10^5	1.07×10^6	1.63
fractions 6-11 from continuous gradient	3.31×10^7	5.95×10^7	90.1

original infectivity. (43)

These data indicated that the centre peak was comprised of purified intact rubella virions. Less than 2% of original infectivity was located in the light density peak. It was not clear what this peak was comprised of. Electron microscopic examination demonstrated the presence of amorphous membranous material. The infectivity in this peak could be due to incomplete virions.

Results of this purification procedure indicated that renografin gradients would permit a high degree of purification with a recovery of intact infectious virions up to 90% of total original infectivity.

IV Isolation and Iodination of Rubella RNA

Virions from high and low interference stocks were purified and examined for the presence of defective particles. The protocol outlined in Figure 9 was followed. In uninfected control samples, comparable fractions were pooled to peak fractions from infected preparations. Control and uninfected preparations were treated in an identical manner. Following iodination infected and uninfected ^{125}I-RNA molecules were eluted off G-25 sephadex columns (Figure 10). Column chromatography indicated that ^{125}I-RNA from infected samples eluted off columns prior to the unreacted iodine. In uninfected samples only unreacted iodine was eluted off

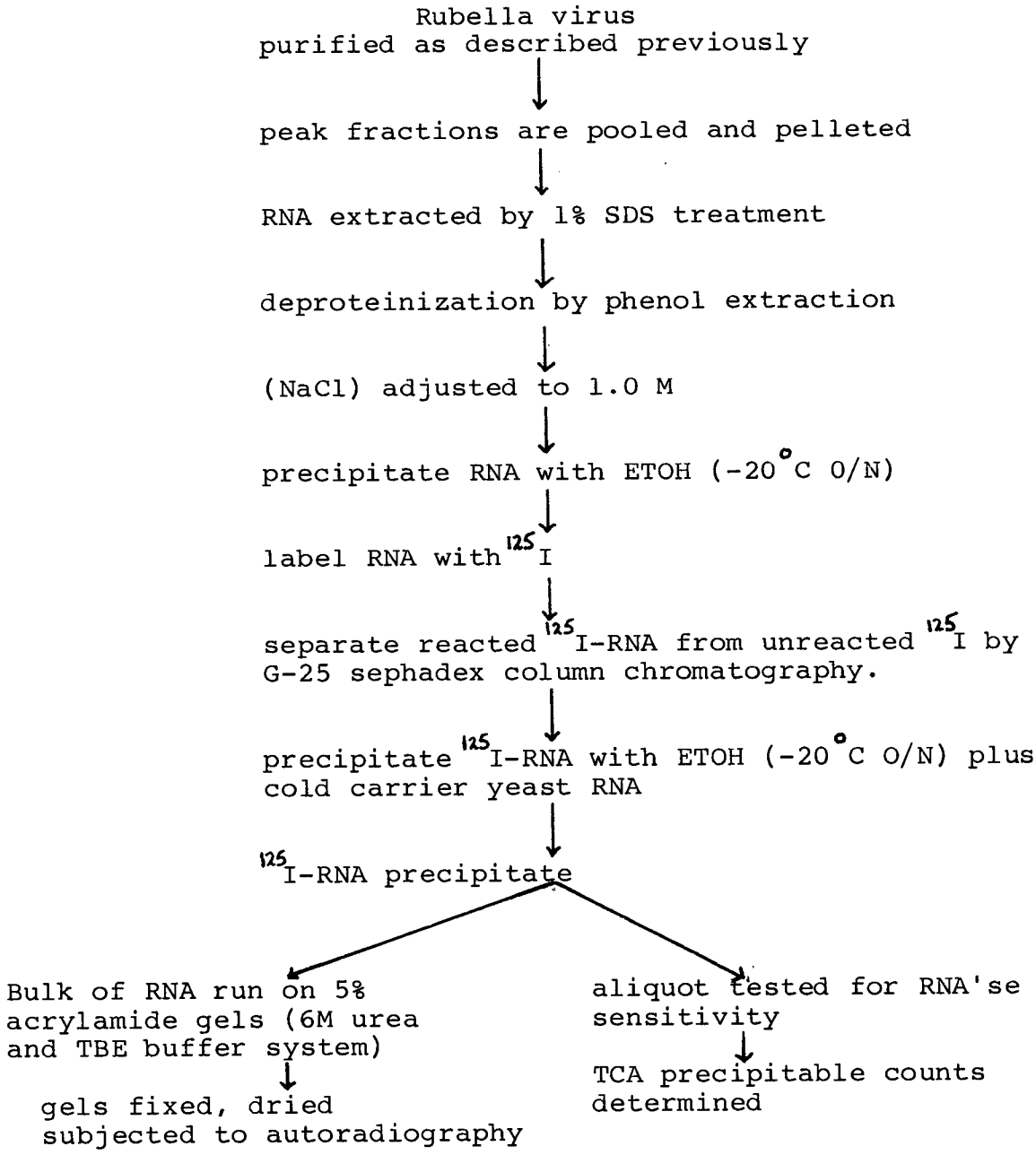

Rubella virus
purified as described previously
peak fractions are pooled and pelleted
RNA extracted by 1% SDS treatment
deproteinization by phenol extraction
(NaCl) adjusted to 1.0 M
precipitate RNA with ETOH (-20°C O/N)
label RNA with 125I
separate reacted 125I-RNA from unreacted 125I by G-25 sephadex column chromatography.
precipitate 125I-RNA with ETOH (-20°C O/N) plus cold carrier yeast RNA
125I-RNA precipitate
Bulk of RNA run on 5% acrylamide gels (6M urea and TBE buffer system)
gels fixed, dried
subjected to autoradiography
aliquot tested for RNA'se sensitivity
TCA precipitable counts determined

Figure 9

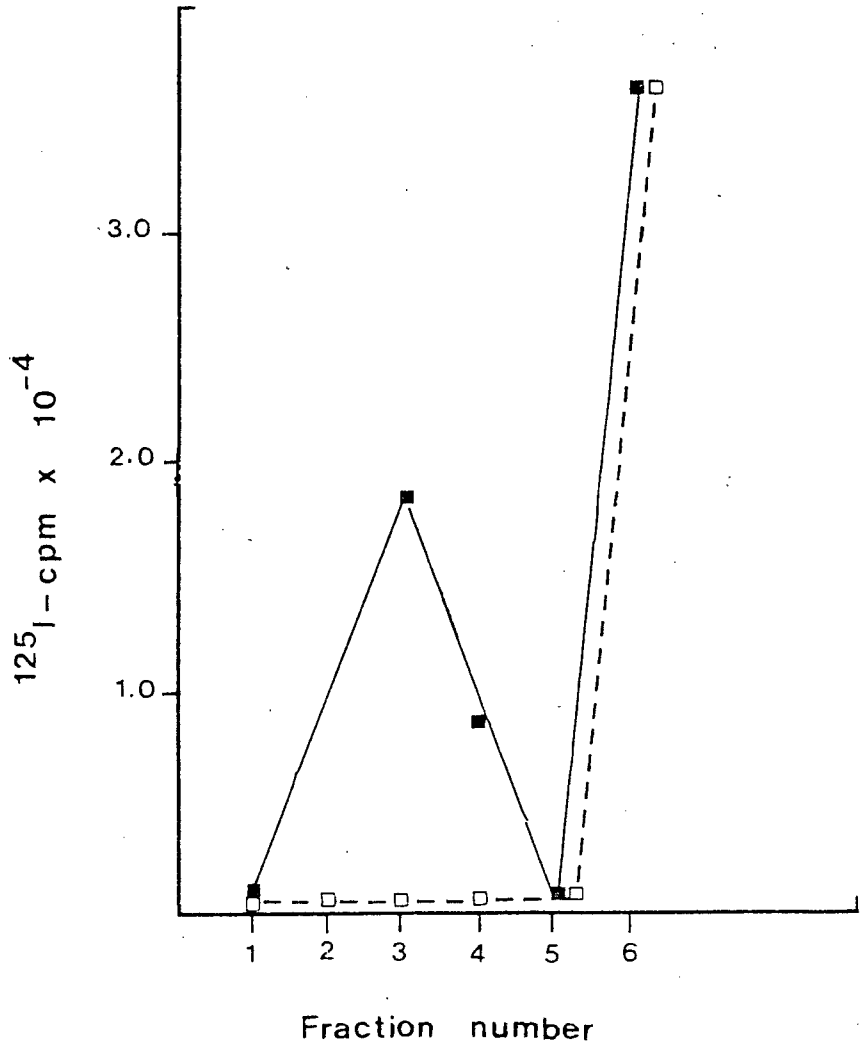


Figure 10. G-25 Sephadex, column chromatography of ^{125}I - rubella RNA

■——■ elution of infected ^{125}I - rubella RNA

□- -□ elution of mock infected ^{125}I-RNA

columns. A small aliquot of the precipitated viral ^{125}I-RNA was removed and tested for RNA'se A sensitivity. In all instances less than 15% of the total counts remained following RNA'se A treatment.

V. Isolation of D.I. Particles from high interference Rubella stocks

High interference and low interference Rubella stocks were obtained. Both stocks were prepared by passaging at a m.o.i. of 0.01. The high interference stock was obtained from passage 2 rubella and had a titer of 4.48×10^4 HAd units/ml. The low interference stock was obtained from passage 6 rubella and had a titer of 5.14×10^4 HAd units/ml. The high interference stock was selected for its ability to reduce viral infectivity by 99.7%. The virions from each stock were purified and analyzed by isopycnic gradient centrifugation (Figure 11).

The low interference stock contained one peak (A) which cosedimented with Sindbis virus at a density $\rho = 1.19$ gm/cc^3. The high interference stock contained virions banding at three densities. The most dense peak (A) cosedimented with Sindbis virus. The two lighter density peaks B and C had densities of $\rho = 1.17$ and 1.15 gm/cc^3 respectively. The virions from each peak were pelleted, the RNA extrated, labeled with ^{125}I, run on polyacrylamide gels and subjected to

Figure 11A - Isopycnic gradient centrifugation of the low interference rubella stock.

■——■ rubella infected preparation

□——□ mock infected preparation

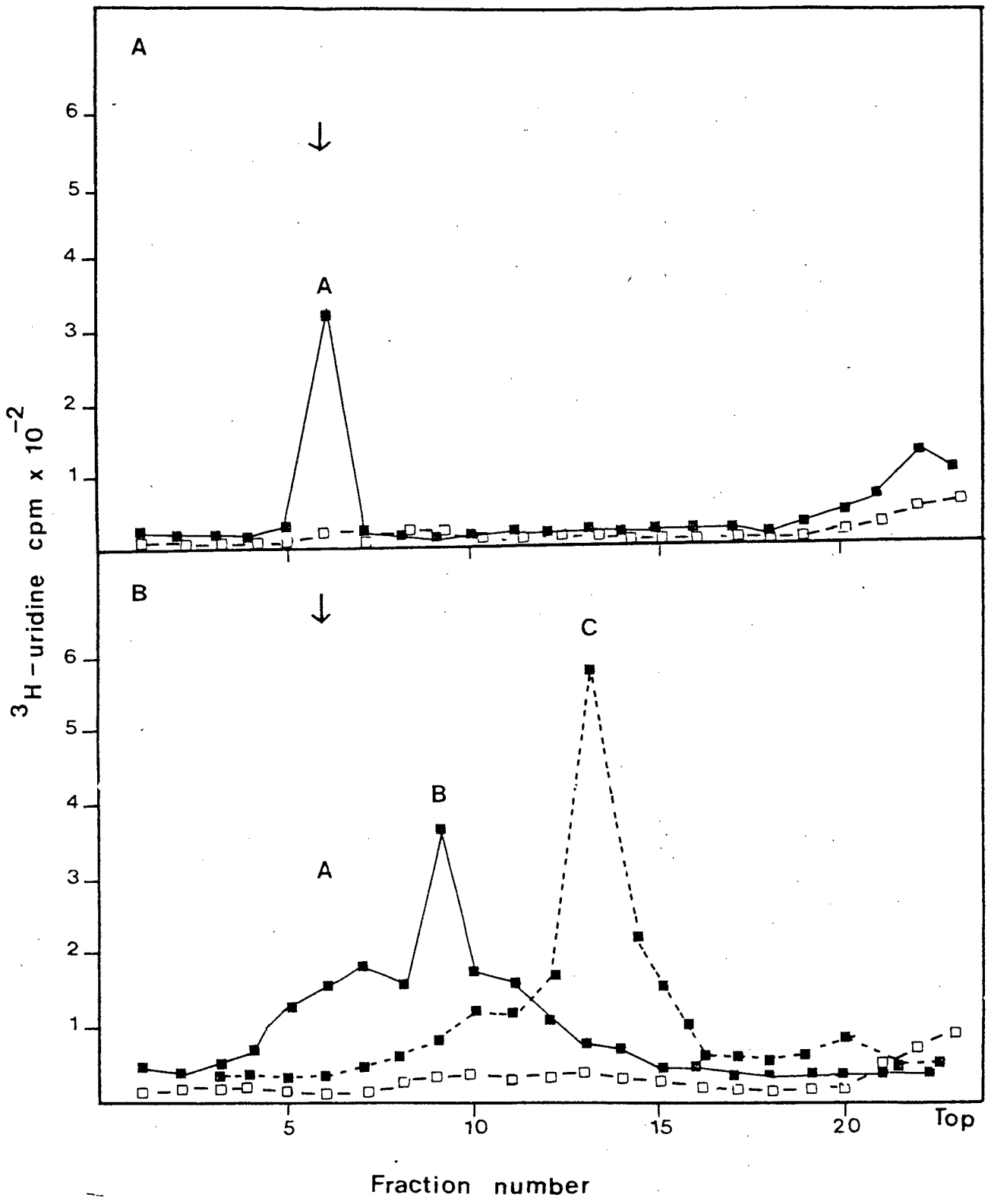
Figure 11B - Isopycnic gradient centrifugation of the high interference rubella stock.

■——■ rubella infected preparation. Peaks A and B were pooled and recentrifuged to achieve better resolution.

■——■ rubella infected preparation. Peak C was pooled and recentrifuged to achieve better resolution.

□——□ mock infected preparation

↓ indicates the position of cosedimented Sindbis virus marker.


A
B
A
B
C
A
B
^{3}H-uridine cpm x 10^{-2}
Fraction number
Top

autoradiography. (Figure 12).

The virions isolated from peak A of the low interference stock contained RNA molecules of 2.95 and 2.80 x 10^6 daltons molecular weight. Repeated experiments confirmed the presence of these two RNA molecules isolated from virions banding at this density. Virions isolated from peak A of the high interference stock contained RNA molecules of identical molecular weight as those isolated from peak A of the low interference stocks. Virions from the lighter density peaks B and C contained RNA molecules of 1.25 and 1.05 x 10^6 daltons molecular weight respectively (Table III).

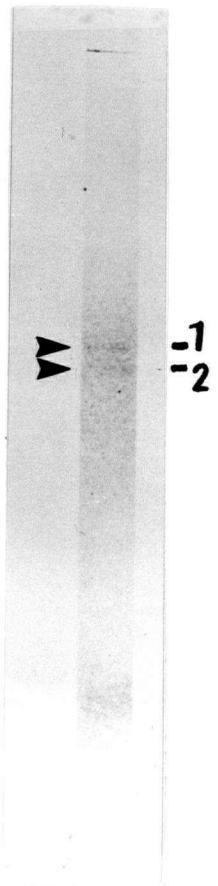
These lighter density particles likely represent true defective particles. The following data would indicate that the defective particles were not artifacts.These lighter density virions were not present in the low interference stock. Virions of only one density were isolated from the low interference stock. The molecular weights of the RNA correlated well with the density of the particles from which they were isolated. The smaller RNA molecules were not degradation products. Single-stranded Sindbis RNA treated in an identical manner electrophoresed to a position equivalent to its theoretical molecular weight. Most important, the molecular weight of the 2.95 x 10^6 dalton RNA contained in virions banding at a density of ρ = 1.19 gm/cc^3 correlated well with the 3.0 x 10^6 daltons reported in the literature.

Figure 12 - Autoradiograms of I-RNA isolated from purified rubella virions of the:

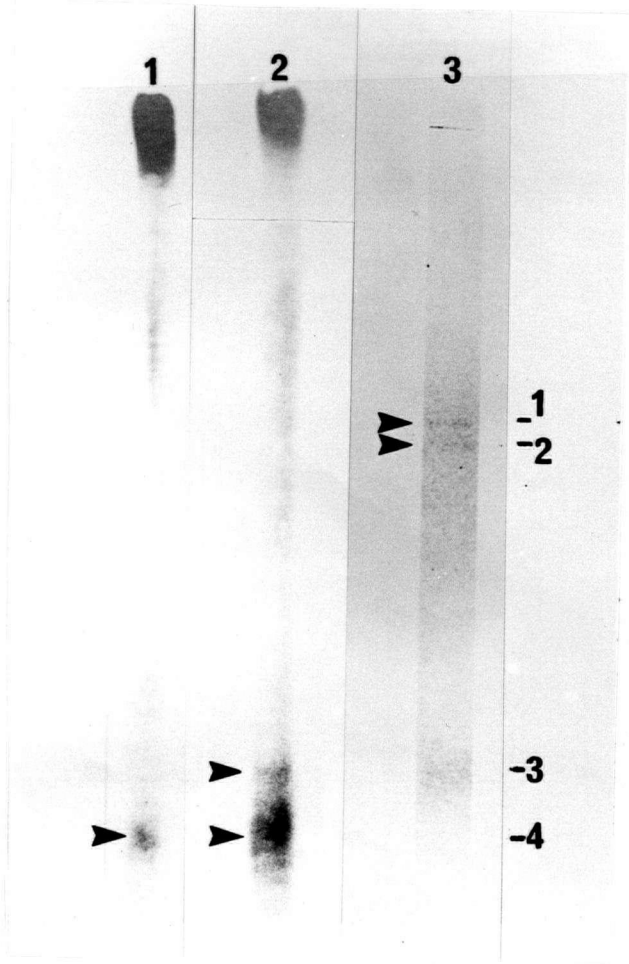
A. - low interference stock. RNA isolated from virions banding at Peak A.

B. - high interference stock. RNA isolated from virions banding at:

Lane 1 - peak C
Lane 2 - peak B
Lane 3 - peak A



12 A



12 B

TABLE III

Molecular weights of Defective Interfering Rubella Virus Ribonucleic Acid

RNA Band (Figure 12)	Source (Peak) (Figure 11)	Molecular Weight x 10^6 daltons	% of Total Genome
1	A	2.95	(100)
2	A	2.80	94
3	B	1.25	42
4	C	1.05	35

The data demonstrated the presence of lighter density defective particles in high interference rubella stocks. These particles were lighter in density, contained smaller molecules of RNA whose size correlated to the density of the particle from which they were isolated, and these defective particles could only be isolated from stocks exhibiting high interference characteristics.

VI The generation of D.I. particles on sequential passaging of rubella following primary isolation.

The presence of lighter density D.I. particles was established in a high interference stock. Rubella was serially passaged to examine the trend of defective particle generation. More specifically the ability of rubella virus to demonstrate a von Magnus effect was determined. A fresh lab isolate was obtained. Rubella titers were always low and variable and a more sensitive method was needed to follow the generation of D.I. particles. The virions from each passage were purified by isopycnic gradient centrifugation (Figures 13 - 17). The RNA was extracted, labeled with ^{125}I-iodine and analyzed by polyacrylamide gel electrophoresis (Table IV). The major species of RNA generated are indicated in Figure 18.

Examination of the RNA molecules extracted from virions at each successive passage revealed that defective particles were rapidly generated. Within two passages of isolation

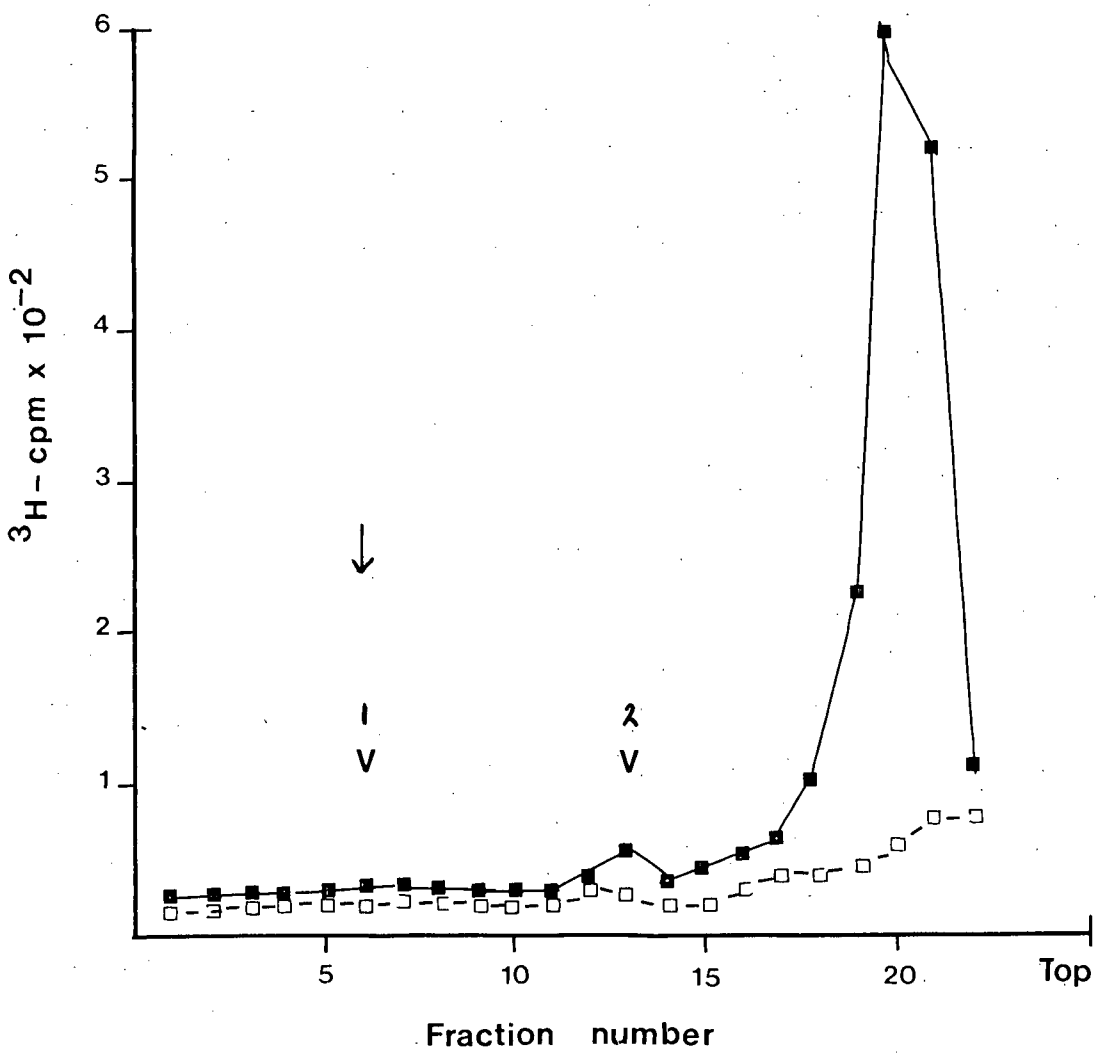


Figure 13 - isopycnic gradient centrifugation analysis of passage 2 rubella

↓ position of standard rubella

■—■ rubella infected

□--□ mock infected

V fractions isolated for RNA extraction

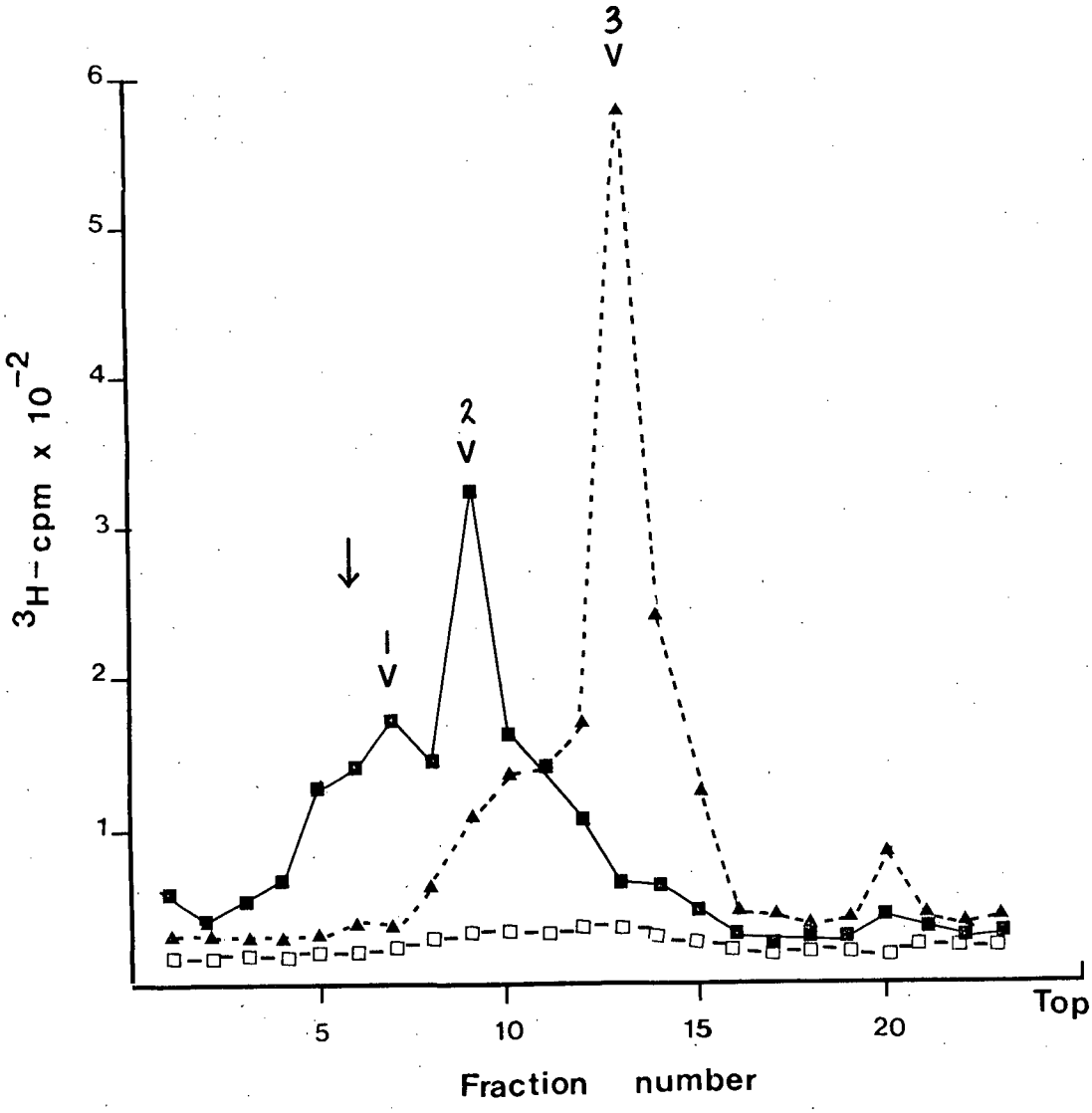


Figure 14 - Isopycnic gradient centrifugation analysis of passage 3 rubella

↓ position of standard rubella

▲---▲ rubella infected - pooled/recentrifuged

■—■ rubella infected - pooled/recentrifuged

□ □ mock infected

V fractions isolated for RNA extraction

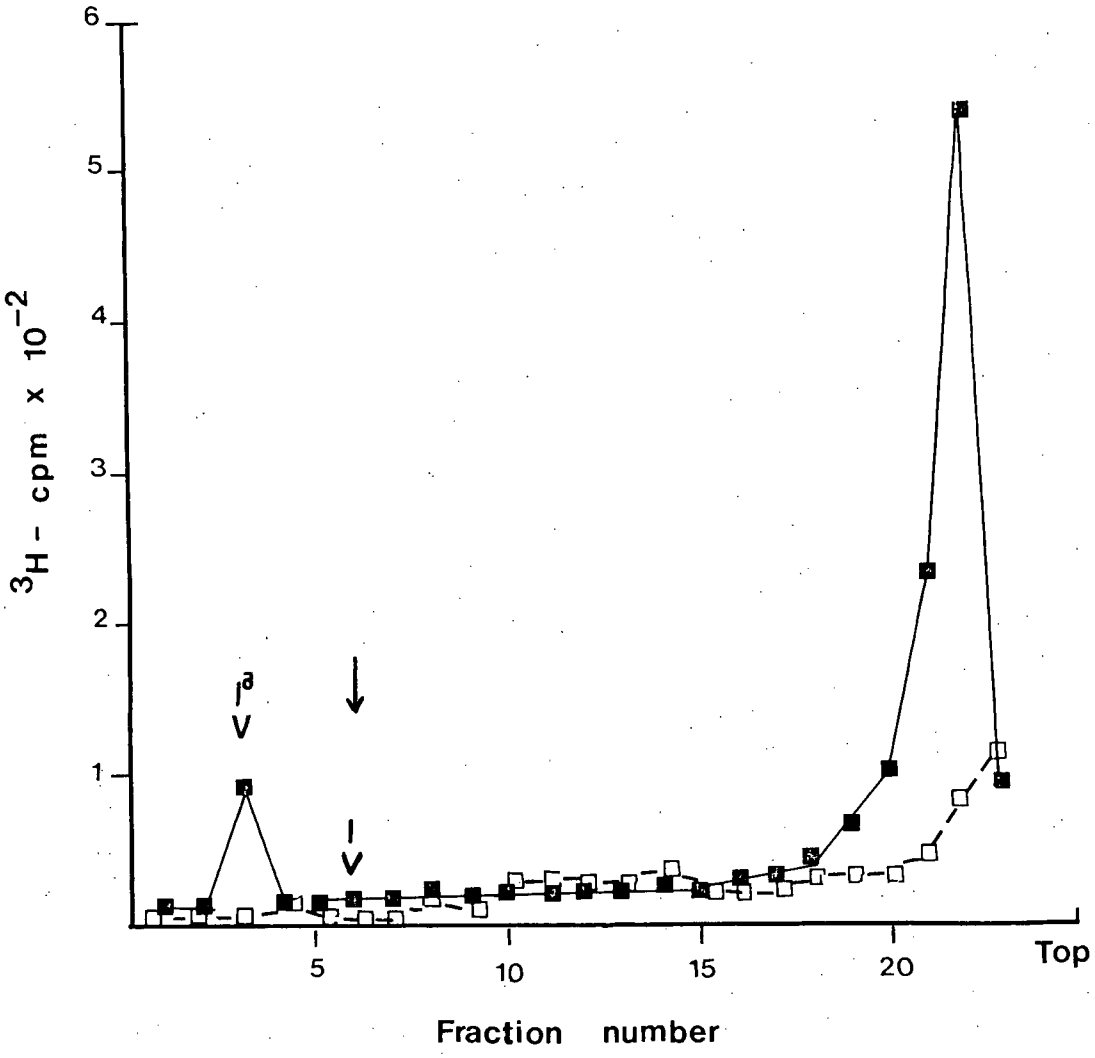


Figure 15 - Isopycnic gradient centrifugation analysis of passage 4 rubella

↓ position of standard rubella

■——■ rubella infected

□- -□ mock infected

V fractions isolated for RNA extraction.

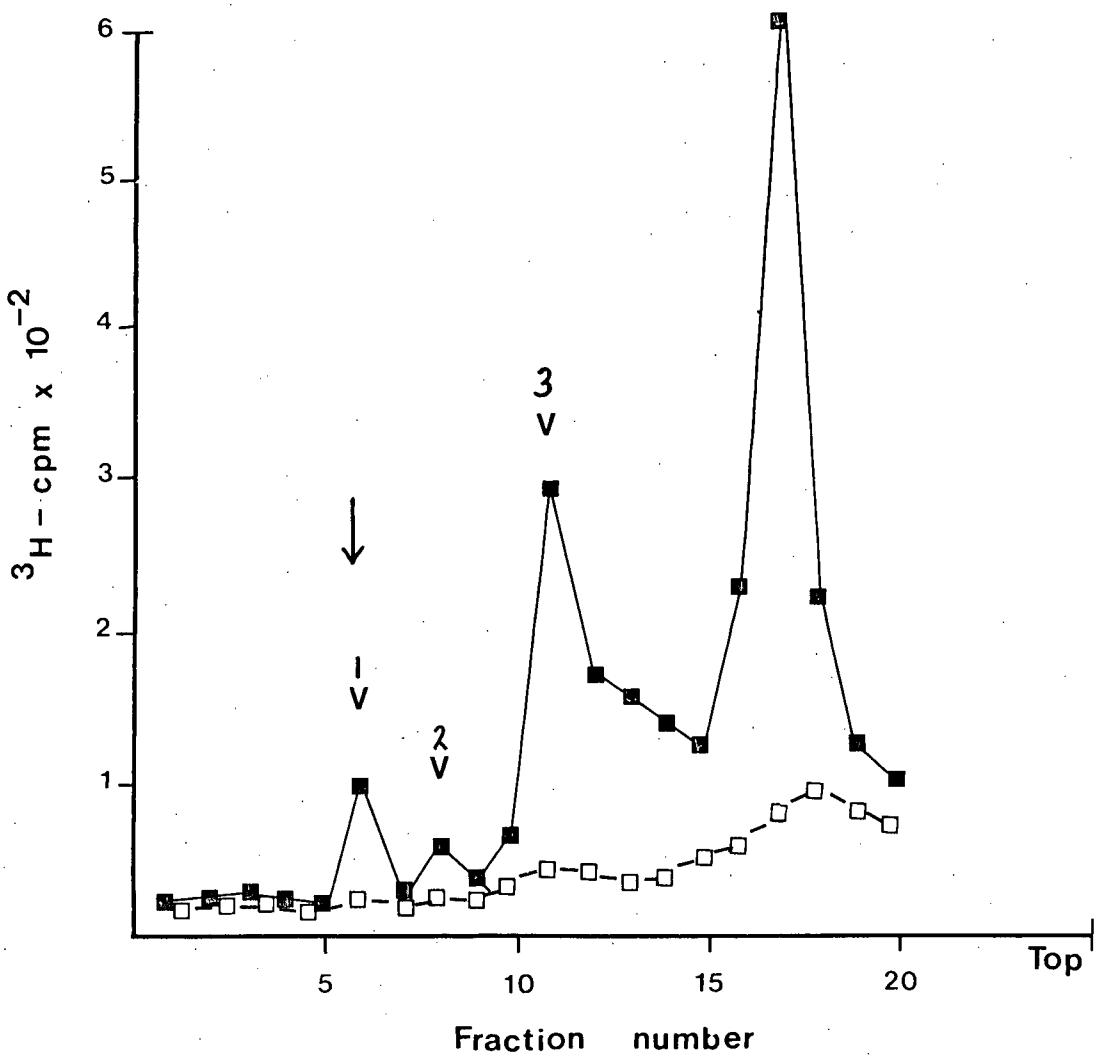


Figure 16 - Isopycnic gradient centrifugation analysis of passage 5 rubella

↓ position of standard rubella

■—■ rubella infected

□–□ mock infected

V fractions isolated for RNA extraction

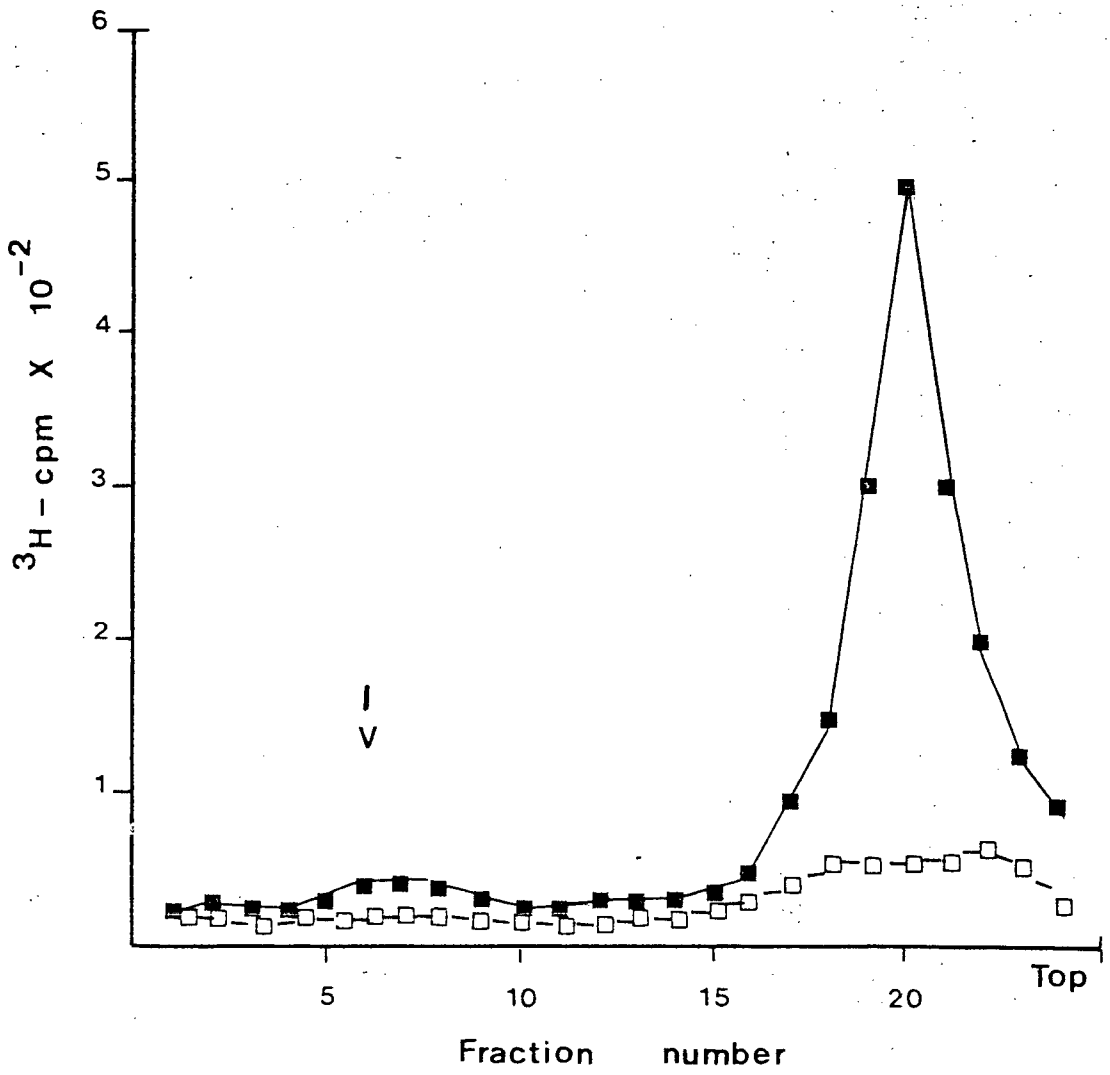


Figure 17 - Isopycnic gradient centrifugation analysis of passage 6 rubella

rubella infected

mock infected

V fractions isolated for RNA extractions

TABLE IV

Rubella Passage Number	Fraction(s) Isolated	Peak Number	Molecular weight of RNA x 10^6 daltons
Standard Rubella	6	1	2.95 2.80
Passage 2	6	1	2.95 2.80
	12 - 14	2	1.30 1.05
Passage 3	6 - 8	1	2.95 2.80
	9 - 11	2	1.25 1.05
	12 - 15	3	1.05
Passage 4	3	1[a]	2.40 1.60 1.00 0.80
	6	1	2.95 2.80
Passage 5	6	1	2.95 2.80
	8 - 9	2	2.30 1.50
	12 - 13	3	not successful
Passage 6	6 - 8	1	2.95 2.80

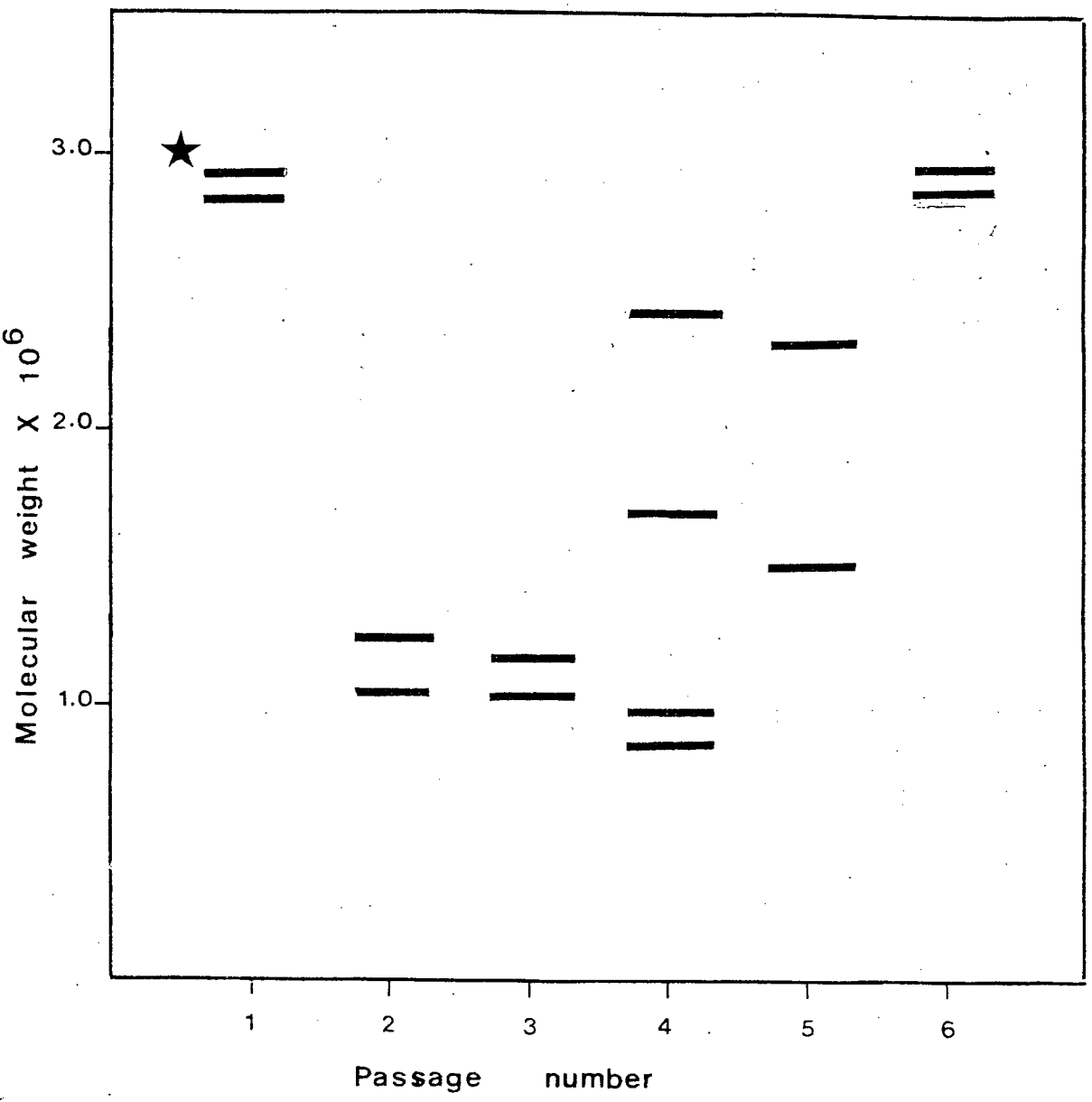


Figure 18 - Major species of RNA generated on sequential passaging of rubella virus. In all passages a small amount of standard RNA could be isolated.

★ expected RNA molecules to be isolated from passage 1 purified virions.

small defective molecules of RNA were generated. The following passages generated virions which had a density greater than ρ= 1.19 gm/cc^3. A diverse range of RNA molecules were isolated from these dense virions. Rapidly thereafter RNA molecules of 2.95 and 2.80 x 10^6 daltons were regenerated.

These results indicated that Rubella conforms to the accepted definition of D.I. particle generation. Rubella demonstrated a rapid cyclic fluctuation in yields of standard and defective virions. These observations suggested that rubella also exhibits the von Magnus effect on continued passaging of the virus.

Discussion

Rubella virus has been titrated using a modified hemadsorption assay. Titration results suggested that virus stocks could be comprised of two populations of virions, both standard and defective where an interference effect was operative or there could be viral aggregation at lower dilutions. The former interpretation was most likely as viral infectivity was reduced by greater than 99% when a high interference stock was mixed with a low interference stock. There is a one hundred fold reduction in virus infectivity when the high interference stock is mixed with the low interference stock. This magnitude of inhibition has been observed in several defective viral systems (15, 19)

Rubella has been highly purified using two renografin gradients which allow 90% recovery of infectivity. The purified virus was found to cosediment with Sindbis virus at a density of $\rho = 1.19$ gm/cc^3 and electron microscopic examination demonstrated the presence of intact rubella virions. The virions were isolated from a broad square peak which may represent poorly separated standard and defective virions.

Virions have been purified from the high interference and the low interference stocks, the RNA extracted and analyzed by polyacrylamide gel electrophoresis.

The low interference stock contained virions banding at one density $\rho = 1.19$ gm/cc^3. These virions contained RNA molecules with molecular weights of 2.95×10^6 and 2.80×10^6

daltons. The high interference stock contained virions banding at a minimum of three densities. Once again virions banded at a density ρ = 1.19 gm/cc^3 and contained RNA molecules of 2.95 x 10^6 and 2.80 x 10^6 daltons. The two classes of lighter density virions banding at densities of ρ = 1.17 gm/cc^3 and 1.15 gm/cc^3 contained RNA molecules of 1.25 and 1.05 x 10^6 daltons respectively.

It would be unlikely that these lighter density virions were artifacts because minimal degradation occurred during purification. Virions banding at only one density ρ = 1.19 gm/cc^3 were consistently isolated from low interference stocks. The lighter density virions could only be isolated from stocks exhibiting high interference characteristics. The presence of defective virions corresponded with the interfering characteristics of the high interference stock.

The virions banding at a density ρ = 1.19 gm/cc^3 contained RNA molecules of 2.95 x 10^6 daltons in molecular weight. This molecular weight corresponded favourably to the molecular weight of 3.0 x 10^6 daltons reported in the literature. The 2.80 x 10^6 dalton RNA molecule may represent a large defective molecule because: (1) this molecule was repeatedly isolated from purified virions of both high and low interference stocks; (2) the single-stranded RNA of Sindbis virus was treated in an identical manner and consistently appeared as a single band on polyacrylamide

gels. Degradation of RNA did not appear to be a problem; (3) An alternate conformation of the 2.95 x 10^6 dalton molecule would seem unlikely under the 6M urea reducing conditions of the polyacrylamide gels.

The virions containing either of these two RNA molecules should have minor differences in their densities. The slight difference in their densities might be enhanced on shallower renografin gradients. If the virions could be separated and purified, the RNA could be analyzed on polyacrylamide gels. The RNA analysis could establish if the 2.80 x 10^6 dalton molecule represented a true defective genome.

The light density virions isolated from the high interference stock contained defective genomes. The 1.25 and 1.05 x 10^6 dalton molecule represent 42% and 35% of the standard genome respectively. The virions from which these molecules were isolated were not degraded virions and there is no reason to believe that these molecules represent degraded 2.95 x 10^6 dalton RNA. There was no degradation of Sindbis RNA and in addition the molecular weight of the defective RNA corresponds to the density of the particle from which they were isolated.

During the course of this investigation several observations were made. Standard rubella stocks free of defectives were never obtained. Low interference stocks contained virions of one density ρ= 1.19 gm/cc^3. Two

molecules of RNA could be isolated from these virions. High interference stocks contained virions of the same density $\rho = 1.19$ gm/cc^3 and in addition virions of two lighter densities. These virions contained smaller molecules of RNA. Only rubella stocks containing these defective particles could elicit interference with a low interference stock. Ideally, one would like to completely separate standard and defective virions prior to conducting mixing experiments. This is not yet possible using the techniques documented here.

Additional observations were less clear. The interference characteristics of a stock could change on passaging. In some instances less than 10^2 HAd units/ml were detectable. Yields of standard virus could not be increased by passaging at low m.o.i's. Similarly defective particles could not be generated at will by simply passaging at high m.o.i.'s.

The presence of both standard and defective particles in rubella populations could well account for the consistently poor yields of virus and for the inconsistent reports of virion density in the literature. This may explain why passage at low m.o.i.'s did not significantly raise viral titers. Some rubella defective particles may always be present and if so some degree of autointerference would always be in effect.

The presence of defective interfering rubella particles has been demonstrated. The generation of defective particles was examined on serial passaging of the virus after primary isolation.

The isopycnic gradient centrifugation profiles indicate that the specific activity of ^{3}H-uridine is too low to detect the virus. Even in the absence of a visible peak of H-uridine incorporation standard molecules of RNA with a molecular weight 2.95×10^{6} daltons could always be isolated from the fractions with a density corresponding to $\rho = 1.19$ gm/cc^{3}. The smaller RNA molecules of 2.80×10^{6} daltons could also be isolated from these virions. These results were not unexpected as a small amount of standard virus must always be present to allow replication of the defective particles.

Different types of defective particles were generated on serial passaging of rubella. Defective particles were generated which were not only lighter in density but were more dense than the standard virion. This latter observation has been reported for Sindbis virus (36). There have been additional reports of virions of increased density containing more than one molecule of RNA. In some cases up to 5 or 6 RNA molecules in multiple copy form have been isolated from these particles (3, 17).

When the RNA molecules from serially passaged virions

were examined a cyclic "von Magnus" effect was observed. This observation has not been previously reported for rubella. Most studies of rubella involved the use of standard lab strains which have been repeatedly passaged over several years. Use of a primary isolate has permitted the visualization of the cyclic "von Magnus" effect. Rubella appears to generate defective particles rapidly. One characteristic of the von Magnus phenomenon is the gradual decrease in the cyclic yields of standard and defective virions. As rubella generates defective particles rapidly it is probable that over several additional passages this cyclic trend would decline and the von Magnus effect would gradually disappear.

The host cell is known to play an important role in the generation of defective particles (21, 29). The factors which allow one cell type to permit defective particle generation and another to prohibit their generation are poorly understood. Little work has been done on the role of the host cell in the generation of defective interfering viral particles.

Several proposals have been put forward to explain the generation of D.I. particles (13, 16, 20) and the means by which they interfer (30, 31). Recent analysis of Sindbis virus and Semliki Forest Virus RNA indicate there is a poly A sequence at the 3' - OH terminus of their genomes (4, 33, 46).

These observations have aided in determining the origins of D.I. particle generation (9). If such a sequence could be located on the standard rubella genome defective RV-RNA molecules could be screened for the presence of this sequence (5). This information may reveal the mechanism by which defective interfering rubella viral particles are generated and ultimately the means by which they interfer.

In summary, rubella virus has been purified from low and high interference stocks. Defective virions could be isolated from high interference stocks which (1) were lighter in density; (2) contained smaller molecules of RNA whose size correlated with the density of the virions from which they were isolated; (3) corresponded to the interfering properties associated with that stock. Virions isolated from low interference stocks contained RNA molecules with molecular weights of 2.95 and 2.80 x 10^6 daltons.

Finally, serial passaging of a primary isolate of rubella and extraction of RNA from the purified virions indicate that rubella may also exhibit a von Magnus effect.

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