EFFECT OF POLYETHYLENE GLYCOL PRECIPITATION AND CENTRIFUGATION ON AVIAN INFECTIOUS BRONCHITIS VIRUS

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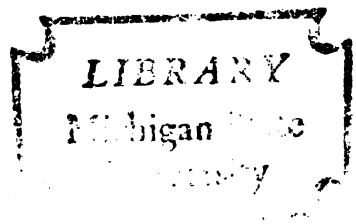

ABSTRACT

EFFECT OF POLYETHYLENE GLYCOL PRECIPITATION AND CENTRIFUGATION ON AVIAN INFECTIOUS BRONCHITIS VIRUS

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The Beaudette strain of avian infectious bronchitis virus (IBV) grown in African green monkey kidney cells VERO in the presence of ^{3}H uridine, precipitated from the cultural medium by polyethylene glycol, and purified by isopycnic centrifugation at 234,000 X g for two hours in linear sucrose gradients was studied by electron microscopy and tested for infectivity. Virus was infectious after precipitation but was not infectious after centrifugation. The viral particles were uniformly round, predominantly electron dense, and lacked surface projections. The amount of radioactivity, protein, and RNA in the purified virus followed a typical growth curve reaching a maximum 51 hours after innoculation. Virus concentrated by negative pressure dialysis and membrane filtration also lacked surface projections but was infectious and otherwise morphologically typical for IBV. It was 16.0 to 22.8 nm larger in diameter than the electron dense particles and more pleomorphic. This suggests that centrifugation at 234,000 X g may have caused damage to the surface of the viral

particles allowing phosphostungstic acid to penetrate into the virions and stain the internal component of the virus. The results suggest that the surface projections are not essential for cellular infection by this strain of IBV.

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A THESIS

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This thesis is dedicated to the memory of

Mrs. Martha "Pat" Spring.

She generously shared her skill and knowledge of laboratory techniques in virology and cell culture with a generation of students. She was both an excellent teacher and a good friend to myself and many other students. Her friendship and cheerful personality are greatly missed.

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INTRODUCTION

Early studies of avian infectious bronchitis were directed to the clinical manifestations and management of the disease and isolation of the etiologic agent in order to produce an effective vaccine. The morphology of avian infectious bronchitis virus (IBV), its physical and biologic properties, growth in tissue and organ culture, and morphogenesis were studied later as suitable techniques developed. These studies provided the basis for classifying and comparing it to other viruses. As new viruses having similar properties or morphology have been isolated, they have been compared to IBV, because a substantial amount of information about IBV has accumulated. In this way, IBV has been the prototype of the coronavirus group (32).

The molecular components of IBV and other coronaviruses, RNA (45,48), and protein (26) have been the subject of recent reports. The adaptation of IBV to a continuous cell line (14,18), obviating primary avian cell cultures and embryonating chicken eggs as cultural media, has facilitated research on IBV. The present work began as an attempt to develop a procedure for concentrating and purifying large quantities of IBV to be used for biochemical and biophysical

studies of viral structure. As this investigation progressed, unexpected alterations in viral infectivity and morphology occurred which might be more significant than the intended study.

LITERATURE REVIEW

Avian infectious bronchitis virus (IBV) causes an acute, highly contagious, respiratory disease in chickens which is of considerable economic importance to the poultry industry due to prolonged decrease in egg production in laying flocks and poor utilization of feed by young chickens (17). It is a member of the coronavirus group which includes viruses causing the human common cold, mouse hepatitis, neonatal calf diarrhea, and others (2,3,27,44). IBV has a diameter of 60 to 120 nm (8,38,39), an ether sensitive lipoprotein envelope (34), and a ribonucleic acid core (1). The specific gravity of the Beaudette strain, as determined by isopycnic density gradient centrifugation, is 1.23 in CsCl and 1.19 in sucrose (16,46). IBV does not agglutinate chicken red blood cells unless it has been modified by either trypsin (13) or ether or selectively isolated by diethylaminoethyl (DEAE) - cellulose chromatography (9).

The Beaudette strain of IBV, arbitrarily designated IBV-42, was originally isolated by Beaudette and Hudson and has been passed hundreds of times in chicken embryos by the allantoic cavity route (5,19). Cultivation of isolates of IBV in primary avian cell or chicken tracheal organ cultures

requires prior initial isolation in, and adaptation to, chicken embryos. In these culture systems IBV produces characteristic cytophatic effects (CPE), the formation of syncytia, in serial passage (15,21). IBV-42 has been adapted to replicate in an African green monkey cell line (VERO) by first making three serial intracerebral passages of the virus in suckling mouse brain (18). Three strains of IBV that had been propagated in chicken embryo kidney cells were adapted to replicate in VERO cells without first passing them in suckling mouse brain (14).

After attachment of the virus to specific receptor sites on the cell surface, infection by IBV occurs with the earliest visible immunofluorescence detected primarily in the perinuclear region but later diffusing throughout the cytoplasm (31). In electron micrographic studies of IBV-42 replicating in the chorioallantoic membrane of embryonating chicken eggs, round electron dense viral particles were observed to form by budding from the membranes into the cisternae of the endoplasmic reticulum (6). The morphogenesis of IBV-42 in chicken embryo fibroblasts (36) and in VERO cells (18) occurs by this same process resulting in syncytia and the formation of plaques in cell monolayers. Maximum virus titers were present at 20 hours after absorption with a decline in titer after that time. The first syncytia were observed by bright field microscopy at 12 hours. At 22 hours large continuous syncytia had formed, and gross CPE was first observed (14).

The characteristic morphologic feature of IBV and the coronaviruses is the pear shaped or bulbous surface projections which are 9 to 11 nm in diameter on the distal end, 20 nm long, and attached to the virus by a narrow neck. Extracellular IBV-42 in negatively stained preparations has a corona of these projections and is quite pleomorphic with respect to size and shape (8). The nature of the surface projections is controversial. Typical IBV particles were present in the 33rd VERO cell passage of three strains of IBV, but particles lacking projections predominated in the preparations of IBV-42 (14). A study of 12 strains of IBV suggests that morphologic variations do exist and are not the result of manipulation. This study also indicated that IBV-42 in freshly harvested, uncentrifuged allantoic fluid did not have surface projections (24). The presence of a corona was not related to the number of egg passages or virulence but did correlate with the ability of the virus to elicit group specific antibody. Studies of the morphogenesis of IBV-42 have demonstrated the surface projections in negatively stained preparations but not in sectioned virus (4,6,47). IBV virions are described as possessing an envelope with a typical three layered unit membrane structure enclosing an internal threadlike component which is seven to eight nm across (4). Electron micrographs of thin sections of chicken trachea and the viral pellet prepared from allantoic fluid, each infected with the same strain of IBV,

reveal inner and outer membranes separated by an electron transparent zone (a unit membrane structure), but surface projections were not present. However, an electron micrograph of negatively stained virus which has typical projections is included in this report (47). Treatment with trypsin removes the surface projections from IBV-42 and destroys its infectivity. Uniformly round particles having electron light edges, electron dense cores, and diameters of approximately 90 nm are present in electron micrographs of negatively stained trypsin treated IBV-42 in allantoic fluid (35).

IBV-42 has been purified by isopycnic density gradient centrifugation in CsCl, which has an inactivating effect on the virus, and in linear sucrose density gradients. Two cycles of centrifugation in sucrose at 109,000 X g increased the specific infectivity of IBV-42 868 fold (46). Infectious virus can also be purified from allantoic fluid by selective elution from a diethylaminoethyl (DEAE) - cellulose column with NaCl solutions (9).

MATERIALS AND METHODS

Viruses

The Beaudette virus, IBV-42, in allantoic fluid and also the 16th through the 20th passages from VERO cells was used in these studies. Stocks of IBV-42 were prepared in VERO cell monolayers (see Cell Culture) by the following method. The growth medium was decanted from VERO cell monolayers in flasks, and the cells were inoculated with cell cultural fluid from the previous passage of virus. The cells were allowed to adsorb virus for 100 min at 37° and were then washed three times with 0.15M phosphate buffered saline (PBS) without Ca++ or Mg++, pH 7.0, containing antibiotics. Medium without serum was added to each flask. Between 49 and 52 hours after inoculation, the medium was decanted and centrifuged at 2,000 X g for 10 min at 4°. For uniformity, all centrifugation for initial clarification of viral preparations was at 2,000 X g and 4° in a PR-6 Refrigerated Centrifuge (International Equipment Co., Needham Heights, Mass.). The supernatant fluid, approximately 3.0×10^4 plaque forming units (PFU)/ml, was dispensed into screw cap vials and stored at -90° until used.

The stock virus in allantoic fluid, 3×10^6 chicken embryo lethal doses 50% per ml, was stored in screw cap vials at -90° until used.

Cell Culture

VERO cells were supplied by Dr. D. L. Croghan, Veterinary Biologics Division, United States Department of Agriculture, Ames, Iowa, as passage 129, but passages 138 through 155 were used in these studies. All cell culture ingredients were obtained from the Grand Island Biological Company (GIBCO), Grand Island, New York. The growth medium for stock cultures consisted of Eagle's minimum essential medium with Earle's basal salts and non-essential amino acids supplemented with 5% fetal calf serum (FCS) and antibiotics (100 U/ml of penicillin, 100 mg/ml of streptomycin, and 6,000 mcg/ml of tylosin tartrate). The medium was buffered to pH 7.4 with filtered 7.5% sodium bicarbonate. Maintenance medium was the same as growth medium except that the FCS was reduced to 2%. The growth medium was replaced with maintenance medium on the third or fourth day. Incubation was at 37° in an atmosphere of 85% relative humidity and 6-8% CO_2 except where otherwise noted.

Confluent monolayers of cells were grown in: (1) screw cap plastic culture flasks, 250 ml (Falcon Plastics, Los Angeles, Calif.), (2) screw cap glass culture tubes, 16 X 125 mm, and (3) cell production roller vessel, 1330 cm^3 (Bellco Glass, Inc., Vineland, N.J.), referred to as a roller bottle.

The stock VERO cultures in flasks were split 1:5 every seven days for maintenance. The maintenance medium

was decanted from the flask, and the cells were washed with 10 ml of PBS. The flask was then inverted to have the cells uppermost. Five ml of 0.25% trypsin (1:250) in GIBCO solution A was added to the flask which was returned to the original position for two min at room temperature to allow a uniform wetting of the cell monolayer by the trypsin solution. The trypsin solution was decanted and the flask was inverted again. After incubation at 37° until the monolayer started to detach from the surface of the flask (15-20 min), the appropriate amount of growth medium was added for the split to be made. The flask was shaken vigorously to disperse the cells uniformly, and FCS was added to a final concentration of 5%. One ml of cell suspension derived from a 1:2 split was seeded in each tube, and 25 ml of cell suspension derived from a 1:5 split was seeded in each flask. A cell monolayer was formed after 24 hours in the tube cultures and after three or four days in the flask cultures.

Prior to seeding the roller bottle with cells, the bottle was kept in an atmosphere of 85% relative humidity, 8-9% CO_2, at 37° for 24 hours. Then 30 ml of growth medium was added, the cap was tightly sealed to retain the CO_2 atmosphere, and the bottle was rolled on a cell production roller apparatus (Bellco Glass, Inc.) for 24 hours at 2 rpm and 37°. This procedure was done to condition the glass surface for attachment of cells. Ten flasks of cells were trypsinized and suspended in 50 ml of growth medium. This

suspension was added to the roller bottle after the growth medium was removed. The bottle was sealed and rotated at 0.25 rpm. After the first 12 hours, the speed was increased to 4 rpm. After each 24 hour period, the medium was decanted and replaced with 50 ml of fresh growth medium. A confluent monolayer of cells had grown after five days.

Tube cultures were used for infectivity tests. The roller bottle was used to grow a large quantity of virus in a small volume of fluid to be further concentrated by ultrafiltration. The flask cultures were used in all other procedures.

Cultivation of IBV-42 in Embryonating Chicken Eggs

Stock virus was diluted 10^{-1} with nutrient broth, and 0.2 ml was inoculated into the allantoic cavity of 10 or 11-day-old chicken embryos (19). All embryos were killed by virus between 23 and 36 hours after inoculation. During this period, the eggs were candled every hour, and the dead embryos were removed and stored at 5^{0} overnight. The allantoic fluid was collected, pooled, and stored at -22^{0} overnight. It was thawed and clarified by centrifugation. The supernatant fluid was stored at -90^{0} until used.

Procedure for Purification of VERO Cell-Adapted Virus

Decant the medium from VERO cells in flasks

↓

Inoculate the cells with stock VERO cell adapted virus 2 ml/flask

↓

After 90 min, wash cell monolayers three times with PBS and add fresh medium not containing serum

↓

Twelve hours after inoculation, add 10 microcuries (mc) of tritium labeled uridine to each flask

↓

At 50 hours post inoculation or later when the maximum cytopathic effect (CPE) is observed, pool the cell culture fluid

↓

Centrifuge at 2,000 X g for 10 min

|

↓ ↓

Supernatant Fluid — cell debris

Add polyethylene glycol (PEG) to final concentration of 7 gm%

↓

Add 10 gm% $NaHCO_3$ to adjust the pH to 9.0 ↓

One hour in an ice bath

↓

Centrifuge at 10,000 X g for 20 min at 4^0

↓ ↓

Supernatant Fluid — Precipitate

Resuspend in 1 ml of 0.5 M PIPES buffer pH 6.8

↓

Layer suspension on a 15-50% sucrose gradient and centrifuge at 234,000 X g for two hours at 4^0

↓

Collect nine drop fractions

↓

Place 0.01 ml of each fraction on a scintillation counting pad

↓

Dry pads for two hours at 60^0

↓

Soak pads in cold 5% trichloroacetic acid (TCA) in an ice bath for 20 min
↓
Rinse twice in acetone
↓
Dry pads in air and place them in scintillation counting vials containing cocktail
↓
Count scintillations/min of each vial using a liquid scintillation spectrometer
↓
Pool the fractions of the gradient which show a peak of radioactivity
↓
Dialyze peak fractions against PBS
↓
Electron microscopy and infectivity tests

To determine the specific gravity of the sucrose gradient containing virus, a duplicate gradient was prepared with only 1 ml 0.5 M PIPES buffer layered on top. This gradient was processed the same as the gradient containing virus. The following paragraphs describe this purification procedure in more detail.

Cell Cultural Medium During Viral Replication

Since large protein molecules in FCS could possibly complicate purification of the virus by competitive binding, the cells were washed three times with PBS after the adsorption period, and then 25 ml of medium which did not contain FCS was added to each flask. Using the light microscope, no difference was observed in either the morphologic CPE during viral replication or the time after inoculation when the maximum CPE was observed in the cells when the cell culture medium included or did not include FCS.

Labelling Virus with 3_H Uridine

An aqueous solution of uridine (New England Nuclear, Boston, Mass.), which had a hydrogen atom or atoms bonded to carbon atom(s) five and/or six of the uridine molecule replaced by tritium 3_H, was diluted with PBS to 100 mc per ml. Ten mc, 0.1 ml, was added to each flask 12 hours after the cells had been inoculated with virus. The 12 hour interval was selected because of the results of the following preliminary experiment. Tritiated uridine was added to cells which had been infected with virus for 9, 12, 15, 18, 21, and 24 hours, respectively. When maximum CPE was observed, the medium was collected and stored in separate containers at -90^0, until virus was purified from each sample by PEG precipitation and ultracentrifugation as outlined above. The radioactivity profiles of the sucrose gradients and peak heights for 12, 15, 18 and 21 hour labelling times were very similar. The 9 hour sample had less radioactivity in the peak, and the 24 hour sample did not have a sharp and distinct peak. The criterion for selecting 12 hours after inoculation, rather than 15, 18 or 21 hours, as the time to add 3H uridine to optimally label virus was convenience in scheduling experiments.

Low Speed Centrifugation and Precipitation

The following preliminary experiment was done to determine whether or not the pellet from clarification centrifugation contained labelled cell-associated virus. The

pellet was suspended in PBS, frozen and thawed three times. This suspension was layered on a 30-60% linear sucrose gradient and centrifuged at 234,000 X g for two hours. A sample of each fraction from the gradient was placed on scintillation counting pads, treated with cold TCA, and counted in a liquid scintillation spectrometer. Each fraction contained less than 18 counts per min (cpm) more than the background level. Hence, the pellet from low speed centrifugation was discarded in later experiments.

A stock solution of 50% w/w carbowax polyethylene glycol 6000 (Union Carbide, New York) and water was used to add PEG to the supernatant fluid to a final concentration of 7 gm% PEG. Concentrations of 3, 4, 5, 6, 7 and 8 gm% PEG were tested and 7 gm% was found to precipitate the most radioactivity. After the PEG and the cell culture fluid were mixed thoroughly in an ice bath, 10 gm% sodium bicarbonate solution was added to adjust to pH 9.0 which is the lowest pH that will result in a visible precipitate after centrifugation at 10,000 X g for 20 min at 4^0. When the pH was raised above 9, the same amount of radioactivity precipitated as at pH 9. The pH is a critical factor in this procedure. The amount of radioactivity which precipitated was the same when the PEG cell culture fluid solution was allowed to stand in an ice bath for one hour, four hours, or overnight. Hence, the solution was allowed to stand in an ice bath for one hour prior to centrifugation. Polson et. al.

(37) first used PEG to fractionate protein mixtures by precipitation.

The PEG cell culture fluid solution was centrifuged at 10,000 X g at 4^0 for 20 min using a RC-2B centrifuge (Ivan Sorvall Inc., Newton, Conn.). The volume of the supernatant fluid was measured, and a sample was prepared for scintillation counting and counted by the method described later for the sucrose gradient fractions. Excess fluid was drained from the precipitate. The precipitate was scraped from the wall of the centrifuge tube using a rubber policeman and suspended in 1 ml of 0.5 M piperazine-N-N' bis [2-ethane sulfonic acid] (PIPES buffer, Sigma Chemical Company, St. Louis, Mo.). It was selected because it has a high buffering capacity at pH 6.8 and the change in pKa per degree C is -0.0085 (23). The pH of the resuspended precipitate was 6.8 using pH paper.

Linear Sucrose Density Gradients

Linear sucrose gradients were made using a lucite block with two cylindrical chambers interconnected by a small channel and having a drain tube in the bottom of one chamber (10). Fifty percent sucrose in 0.01 M PIPES buffer was placed in the chamber having the drain tube, and 15% sucrose in 0.01 M PIPES buffer was placed in the other chamber. The chamber having the drain tube was stirred by a motor driven screw. An 18 gauge hypodermic syringe needle was fitted to the the drain tube, and the gradient was drained into a

cellulose nitrate centrifuge tube (Beckman Instruments, Inc., Palo Alto, Calif.). The precipitate suspension was layered on one gradient, and an equal volume of 0.5 M PIPES buffer was layered on the other gradient. Before the precipitate suspension was layered on the gradient, 0.01 ml of the suspension was deposited on a scintillation pad (Arthur H. Thomas Company, Philadelphia, Penn.). The total volume of each gradient was approximately 5 ml.

Ultracentrifugation, Fractionation, and Scintillation Counting

Sucrose gradients were centrifuged at 234,000 X g for two hours at 4^0 using either a Beckman model L3-50 or L3-65B ultracentrifuge and a SW50.1 rotor (Spinco Division of Beckman Instruments, Inc., Palo Alto, Calif.). The rotor was decelerated with braking, and fractions were collected by piercing the bottom of the centrifuge tube with a double beveled canula. Nine drop fractions of the viral gradient and 27 drop fractions of the buffer gradient were collected. A sample of 0.01 ml from each fraction of the viral gradient was deposited on a scintillation pad. The pads were dried at 60^0 for two hours, immersed in cold TCA in an ice bath for 20 min, immersed in two changes of reagent grade acetone, and then air dried for one hour. This is a modification of a method developed by Schmidt and Thannhauser who used TCA to precipitate desoxyribonucleic acid (DNA) and ribonucleic acid (RNA) from minced animal tissues (42).

The pads were placed in scintillation counting vials which contained 3 to 5 ml of cocktail containing 0.50 gm/L 1, 4-bis [2-(4-methyl 5-phenyloxazolyl)] benzene, and 6.02 gm/L 2, 5-diphenyloxazole (Packard Instruments, Inc., Downer's Grove, Ill.) in toluene. The background of the vials and cocktail was counted before the pads were inserted into the vials. Only vials which had a background count of less than 25 cpm were used. The average cpm of all these vials was considered the background cpm, and this was subtracted from the cpm of each vial containing a pad. The empty vials were counted for one min each to identify vials with high cpm for removal and counted five min each to determine the background using a Packard Tri Carb Liquid Scintillation Spectrometer either model 3330 or 3320 (Packard Instruments, Inc.). A 0.01 ml sample of the suspension of the pellet from the viral sucrose gradient in 1 ml of 0.5 M PIPES buffer was deposited on a scintillation pad which was treated and counted by the same procedure that was used on the samples of the viral gradient fractions.

The refractive index of each fraction of the gradient that contained only buffer was determined using a Precision Refractometer (Bausch & Lomb Optical Co., Rochester, N.Y.) with conversion of these data to specific gravity (49).

Negative Pressure Dialysis of Allantoic Fluid

Virus in allantoic fluid was concentrated with a negative pressure dialysis apparatus. A vacuum hose was fitted to the side arm of a 3L vacuum flask that contained approximately 800 ml of 0.1 M PBS. Holes were drilled through a rubber stopper for the flask. One end of cellulose dialysis tubing (Sargent-Welch Scientific Co., Detroit, Mich.) was stretched to fit over plastic tubes, which were then forced into the stopper, and the other end was knotted and immersed in the PBS. Allantoic fluid -as continuously added to each dialysis tube via the plastic tubes. The apparatus was kept at 4°, and the vacuum was continuously applied except when the PBS was changed every 12 hours. After 72 hours, 640 ml of allantoic fluid had been concentrated to 5.6 ml.

Normal allantoic fluid from 12-day-old embryonating chicken eggs was concentrated approximately 100 fold by dialysis using the same method as for the virus in allantoic fluid.

Ultracentrifugation of Concentrated Allantoic Fluid

Allantoic fluid which had been concentrated by negative pressure dialysis was used in the following procedure. One ml of concentrated allantoic fluid containing virus and 1 ml of concentrated normal allantoic fluid were each layered on separate 5 ml, 15-50% sucrose gradients. The gradients were processed as previously described. Fractions

9 through 11, 12 through 14 and 15 through 17 from each gradient were collected in separate tubes, dialyzed and examined by electron microscopy.

Inoculation of the Roller Bottle Culture

The medium was decanted from a roller bottle culture of VERO cells, and the cells were inoculated with 10 ml of stock VERO cell adapted virus. The bottle was rolled at 2 rpm for 70 min at 37^0, and then the cells were washed three times with Earle's basal salts solution containing antibiotics. Fifty ml of medium without serum was added to the bottle which was rolled at 4 rpm for 51 hours at 37^0. At this time, the maximum CPE was present. The medium was decanted and centrifuged at 2,000 X g for 12 min. The supernatant fluid was concentrated by ultrafiltration.

Filtration of VERO Cell Adapted Virus

Fifty ml of cell culture fluid containing virus was placed in an Amicon model 52 stirred cell containing a PM 10 DIAFLO membrane which retains molecules weighing more than 100,000 daltons (Amicon Corporation, Lexington, Mass.) at 4^0. Forty pounds per square inch pressure was applied to the cell using compressed nitrogen. After approximately 1½ hours, the 2.5 ml of retentate fluid, a 20 fold concentration, was removed from the cell, examined by electron microscopy, and tested for infectivity in VERO cells.

Infectivity Testing

Four-tenths ml of the retentate fluid was diluted with 7.6 ml of maintenance medium to restore the fluid to its original concentration. This solution was serially diluted 10^{-1} to 10^{-6} in maintenance medium. For determining viral infectivity, triplicate culture tubes of VERO cells were each inoculated with 1 ml per dilution. These tubes were examined for CPE several times each day but scored for infectivity 55 hours after inoculation. Three tubes of uninfected cells served as a control.

Determination of Protein Concentration of Cell Culture Fluid During Viral Replication

Flasks of VERO cell monolayers were inoculated with virus, and 12 hours later, 3_H uridine was added to each flask as described previously. At 30, 37, 44, 51, and 58 hours after inoculation, the culture medium was decanted and clarified by centrifugation. The supernatant fluid was stored at -90^0 until the following determination was done. One ml of this fluid from each time period was thawed and diluted 1:2 with distilled water, and its protein content determined by the method of Lowry et. al. (29) as modified by Campbell et. al (12). The protein standard was bovine serum albumin (BSA) crystallized and lyophilized (Sigma Chemical Company). Phenol reagent (Folin-Ciocalteu) was used (Harleco Hartman-Leddon Company, Philadelphia, Penn.). The addition of reagents and the reading of optical density (OD) were timed to

allow 30 min for color development in each tube. The OD of each reagent blank, standard and sample was determined against a distilled water blank with a Beckman DB spectrophotometer (Beckman Instruments, Inc.) with standard, 1 cm cells at a wavelength of 500 mu. Each reagent blank, standard and sample was done in duplicate. Two reagent blanks were read first, then the standards and samples and finally two other reagent blanks. The average OD of the four reagent blanks was substracted from each duplicate standard and sample. The OD of each duplicate standard and sample was averaged. A calibration curve based on the standards was used to determine the mgs of protein as BSA per ml of cell culture fluid in each sample.

Recovery of Incorporated 3H Uridine from Cell Culture Fluid by TCA Precipitation and NaOH Hydrolysis

The method of Schmidt and Thannhauser was used (42). Five ml of the clarified cell culture fluid from the 30, 37, 44, 51, and 58 hour samples was thawed. While the samples were in an ice bath, 0.55 ml cold 50% TCA was added to each sample. Twenty min was allowed for precipitation and the samples were centrifuged at 500 X g for 15 min at 4^0 with an International PR-6 Centrifuge. The precipitates were washed and centrifuged twice with 5 ml of cold 50% TCA. Each precipitate was suspended in 1.0 ml of 0.1 N NaOH and kept in an ice bath for 15 min. This suspension was centrifuged at 2,000 X g for 12 min, and 0.1 ml of the

supernatant fluid from each sample was deposited on a scintillation pad. The pads were dried, placed in vials containing cocktail and counted as previously described. The background was subtracted from the counts of each sample, and these data were converted to cpm of TCA precipitable material which was hydrolyzed by 0.1 N NaOH per ml of cell culture fluid.

Electron Microscopy

Dialysis

All specimens from sucrose gradients were dialyzed to remove the sucrose. The fraction having the greatest cpm and the fraction on either side of this fraction were pooled and considered to be the viral peak. These three fractions were placed in a section of cellulose dialysis tubing (Sargent-Welch Scientific Co., Detroit, Mich.) which was knotted at one end. Then the other end was knotted, and the tube was placed in a beaker containing 200 ml of cold PBS and a Teflon-coated magnetic stirring bar. The contents of the beaker were stirred, and the PBS was changed five times at one to two hour intervals. This dialysis was done at approximately 5^0.

Pseudoreplica Technique

This method is a slight modification of a technique used by Sharp (43).

Special Agar-Noble (Difco Laboratories, Detroit, Mich.), 2%, in distilled water was poured into a glass petri dish to solidify into a layer approximately 5 mm thick. The agar was poured and used in the same day. For each grid a block of agar, 1 cm X 1 cm, was cut and placed on the end of a glass slide. A drop of dialyzed virus was placed on the surface of the agar. Several drops of 0.75% Parlodian (nitrocellulose) in amyl acetate were spread over the surface of the block, and filter paper was pressed to the edges of the block to blot excess fluid. Immediately after the film had dried, each edge of the block was cut with a new razor blade. A 0.5% phosphotungstic acid (PTA) solution was prepared and neutralized to pH 7.0 with KOH pellets. When the glass slide and agar block were very gently dipped under the surface of the PTA solution in a petri dish, the pseudoreplica film floated from the agar and onto the surface of the PTA solution. A 200 mesh copper grid was placed on the film. The flat end of a round brass rod, 1 mm in diameter more than the grid, was pushed straight down on the grid. This resulted in the film being wrapped over the end of the rod. The stained virus, the film, and the grid adhered to the rod as it was lifted from the PTA solution. The grid was dried overnight under a cover while on the end of the rod.

This technique was somewhat more complex than the routine procedure used to negatively stain virus particles.

It was used, because a vacuum evaporator was not required to coat the grids with carbon, and the particles were distinct against a uniformly stained background.

Preparation of Grids Having a Supporting Film

The film supporting viruses on grids must have high transparency for electrons, adequate strength,and resistance to decomposition and shrinkage when irradiated. Thin plastic films are electron transparent and have adequate strength but tend to decompose and shrink when irradiated. By coating plastic films with a very thin layer of carbon, decomposition and shrinkage are reduced. The following method was used to prepare grids covered with a film of Parlodian and coated with carbon (25).

A beaker shaped vessel, having a perforated shelf, that can be lifted out of the vessel, and a drain which was fitted with a section of rubber tubing and a clamp, was filled with distilled water. A round piece of filter paper, 10 cm in diameter, was placed on the shelf underneath the water. Copper grids were cleaned in Freon, dried and placed under the water on the filter paper. Three or four drops of 0.5% Parlodian in amyl acetate were spread on the surface of the water. In order to clean the surface of the water, this Parlodian film was allowed to dry and then removed with a toothpick. Another film was formed in the same manner. The most uniform part of this film was aligned over the grids.

The water was slowly drained from the vessel, until the film came to lie on the grids. The shelf was removed, and the film, grids, and filter paper were air dried overnight at 20^0 under a cover.

The grids were placed, film side up, on a glass slide in a vacuum evaporator (Denton Vacuum, Inc., Cherry Hill, N.J.). After evacuating the chamber, an arc was produced between two carbon rods, and the grids were lightly coated with carbon. These grids were used in the following two negative stain techniques.

Negative Staining

One drop each of dialyzed fractions from sucrose gradients was placed on a Parlodian carbon coated grid and allowed to dry. A drop of 2% PTA, pH 6.1, was placed on a grid. After 30 sec., the PTA solution was blotted from the grid by touching the edge of the grid with a piece of filter paper. A drop of distilled water was placed on the grid and then blotted off with filter paper. The grid was air dried under a cover.

Negative Staining Using a Nebulizer

This technique was used to obtain optimal visualization of individual virus particles. Dialyzed fractions from sucrose gradients and the retentate fluid from an ultrafiltration cell were used. The following reagents were gently mixed together in a standard glass nebulizer

(Vaponefrin Company, New York): 18 drops of distilled water, four drops of 4% PTA pH 6.8, one drop of 0.1% BSA Cohn fraction V in distilled water, and one drop of the virus sample. A Parlodian carbon coated grid was held 2 or 3 cm from the muzzle of the nebulizer, and the nebulizer bulb was firmly squeezed once. The grid was allowed to dry. The BSA fraction was added to evenly disperse the particles. Care was taken to avoid denaturing the BSA by very gently mixing it into solution, as this protein affects the surface of the particles to promote spreading and separation. The virus samples were diluted in this procedure, and the nebulizer was used to produce a fine dispersion of the particles on each grid. The PTA stain provides the best contrast at the edge of each particle when the particles are well dispersed. This technique (11) is a modification by A.E. Ritchie (40), National Animal Disease Laboratory, Ames, Iowa.

Preparation of Thin Sections

VERO cell virus which had been purified by PEG precipitation and ultracentrifugation, and virus in allantoic fluid which had been concentrated by negative pressure dialysis and purified by ultracentrifugation were each dialyzed, and several drops of each sample were negatively stained. The remainder of each sample was pelleted by ultracentrifugation, embedded in plastic, sectioned stained, and then examined. The detailed procedure is given below.

Linear sucrose density gradients from 36.23% sucrose (specific gravity 1.157) to 15% sucrose in 0.01 M PIPES buffer were prepared. This range was chosen, because in purification experiments the specific gravity of the viral peak was greater than 1.163. The virus samples were layered onto 4 ml gradients and centrifuged at 234,000 X g for 100 min at 4^0. The ultracentrifuge was stopped with braking, the sucrose solution was gently poured from each tube, and then the remaining droplets of sucrose were wiped from the walls of the tubes. The end of each tube was immersed in a 42^0 water bath, and 0.5 ml 2% Special Agar-Noble in 0.01 M PIPES buffer at 42^0 was added to each tube. The pellet in each tube was broken up and suspended in the agar by drawing the agar solution up and down in a warm pipette. Drops of the virus agar suspension were deposited on glass slides which were lying on an ice bath. The agar solidified and was cut into cubes which were about 1 mm on each side. The cubes were transferred to glass vials which were also on ice. Two ml cold 2% glutaraldehyde, E. M. grade, in 0.1 M sym-collidine (2, 4, 6-trimethylpyridine) buffer (Polysciences, Inc., Warrington, Penn.), which was more than sufficient volume to cover the cubes, was added to each vial. The collidine buffer was neutralized with HCl to pH 7.4, and the pH remained 7.4 after 2% glutaraldehyde was added (7). This fixative solution had 350 milliosmols per Kg. The cubes remained in this solution on an ice bath for

eight hours, then they were washed three times in cold collidine buffer. Osmium tetroxide, 1%, in collidine buffer was added to each vial, and the cubes remained in this solution for two hours at room temperature (41). The following series of solutions was added to each vial:

Dehydrating Solution	Dehydration Time
50% ethanol (in distilled water)	5 min
50% ethanol " " "	5 min
70% ethanol " " "	5 min
95% ethanol " " "	5 min
100% absolute ethanol (undenatured)	5 min
100% absolute ethanol "	5 min
100% absolute ethanol "	15 min

To remove the ethanol, the cubes were mixed in three changes of propylene oxide with five min of mixing during each change. The cubes were infiltrated with an Epon mixture (28) consisting of 49.12 gm Epon 812 embedding medium (a glycerol based, aliphatic, epoxy resin, Polysciences, Inc.), 30.48 gm deodecenyl succinic anhydride, and 20.40 gm nadic methyl anhydride. The samples were infiltrated with the Epon mixture according to the following schedule:

Infiltrating Mixture	Mixing Time
Two parts propylene oxide and one part Epon mixture	30 min
One part propylene oxide and two parts Epon mixture	3 hours
Epon mixture	10 hours

The samples were transferred to the final embedding medium which consisted of 50.00 gm Epon mixture and 0.7 ml of an amine accelerator, 2, 4, 6-tri (dimethylaminomethyl)-phenol (Polysciences, Inc.). After mixing thoroughly, the cubes were placed at the bottom of BEEM capsules. The capsules were filled with the final embedding medium which was polymerized at 45^0 for 17 hours followed by 60^0 for ten hours (30).

The hardened blocks were removed from the capsules, and each block was trimmed with a razor blade to give a sectioning square 0.1 mm on each side. Sections approximately 400Å thick were cut by Mr. H. S. Pankratz using an Ultrotome III (LKB, Stockholm, Sweden) and a diamond knife (E. I. duPont de Nemours, Wilmington, Del.). The sections were placed on Freon-cleaned 200 mesh copper grids and stained for 30 min in 2% aqueous uranyl acetate followed by 15 min in 0.5% aqueous lead citrate (22). The sections were immersed twice in water and dried under a cover. They were examined in a Philips EM 300 electron microscope (Eindhoven, The Netherlands) at an instrumental magnification of 67,000.

Electron Microscopy of Negatively Stained Specimens

Negatively stained specimens were examined in a Hitachi Hu-11 electron microscope (Tokyo, Japan) at instrumental magnifications between 58,400 and 83,300.

RESULTS

Purification of VERO Cell Adapted IBV-42 by PEG Precipitation and Sucrose Density Gradient Centrifugation

The maximum radioactivity occurred in fraction 12, specific gravity 1.164 (Figure 1). The gradient used to measure specific gravity had an approximately linear decrease in specific gravity from 1.235, at the bottom of the tube, to 1.115. In three additional similar experiments, the specific gravities of the peak fractions were 1.169, 1.176 and 1.178. The mean specific gravity was 1.172 for all four experiments with a standard deviation (σ) of 0.00646. According to Ellis (20), the specific gravity of chicken embryo kidney cell propagated virus was 1.18 to 1.20 in linear sucrose gradients using infectivity to locate the fractions containing virus.

Pooled fractions 11, 12, and 13 contained two morphologically different particles (Figure 2). The most numerous particles were round and electron dense (appearing dark) but had an electron light ring surrounding the dense area. The electron light particles were more pleomorphic, and the edges were distinct. The pear-shaped or bulbous surface projections described by Berry et. al (8) were not

seen on any particles. The pooled fractions were not infectious in tube cultures of VERO cells using CPE as the criterion for infection.

FIGURE 1.--Sucrose density gradient centrifugation of the PEG precipitate from virus infected VERO cells. Twelve hours after inoculation, ^{3}H uridine was added to the cell culture medium. The medium was collected when the CPE was maximum. Centrifugation was at 234,000 X g for two hours at 4^{0}. The specific gravity was determined by measuring the refractive index of fractions of a duplicate gradient not containing virus.

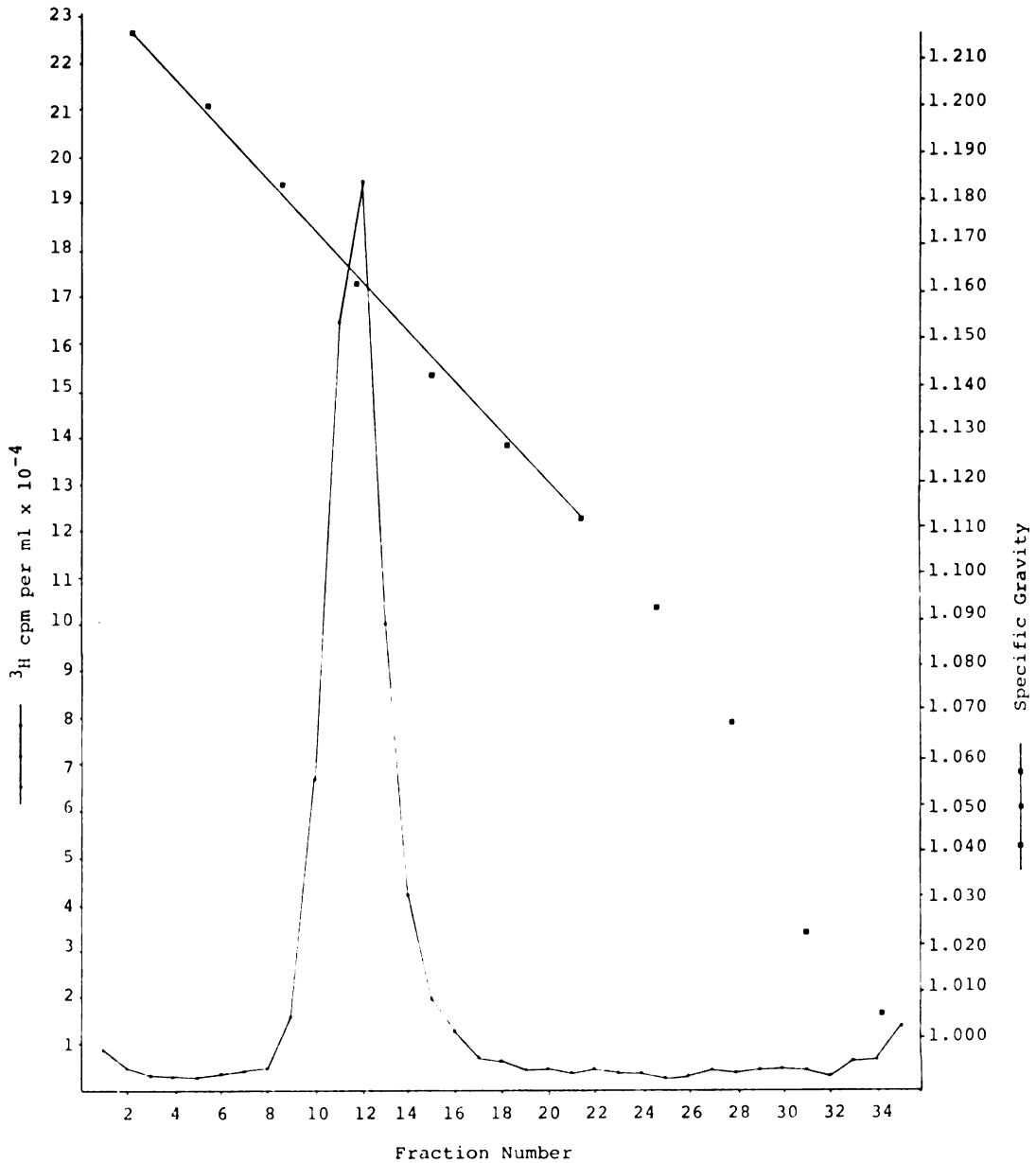
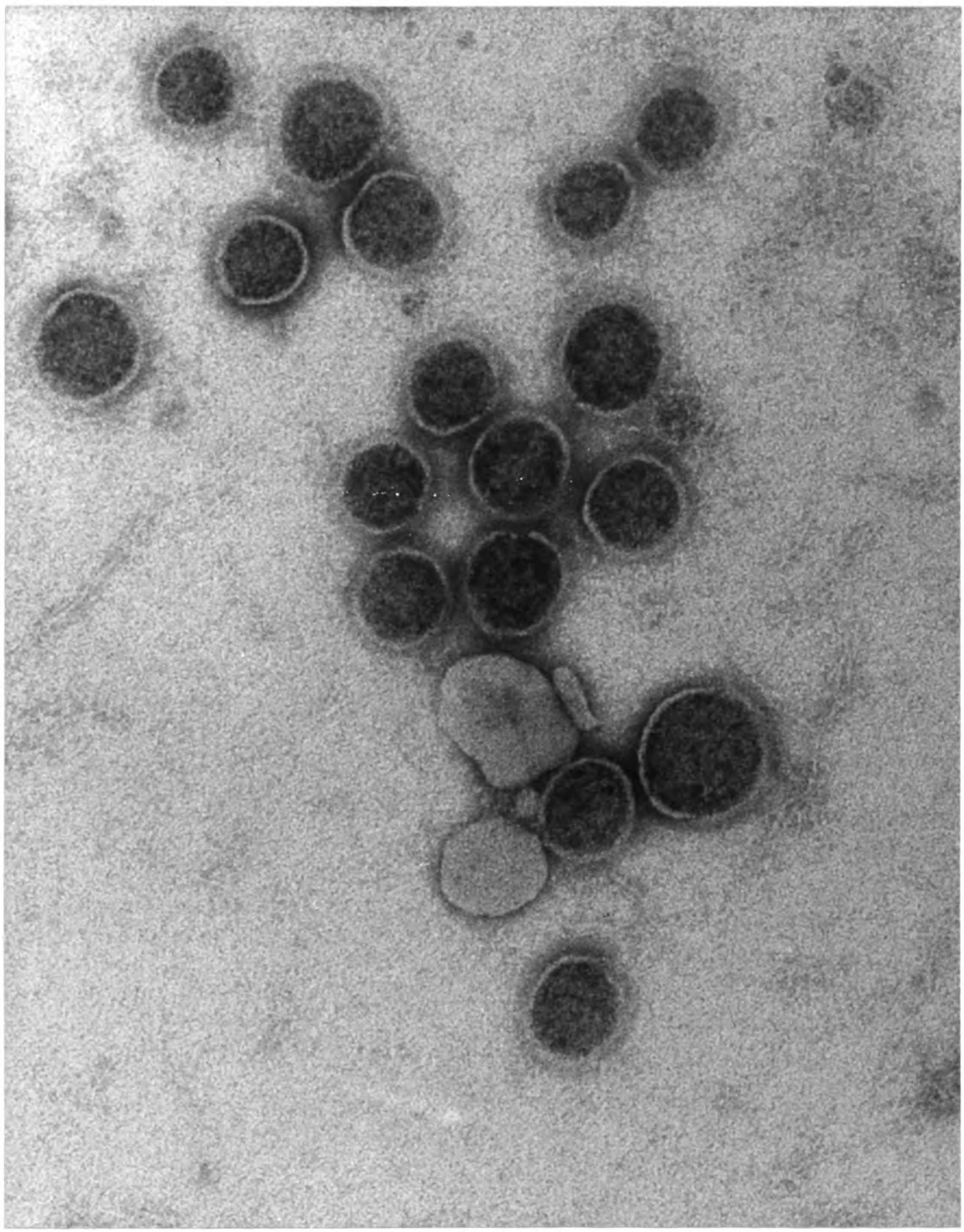

^{3}H cpm per ml x 10^{-4}
Specific Gravity
Fraction Number
23
22
21
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
1.210
1.200
1.190
1.180
1.170
1.160
1.150
1.140
1.130
1.120
1.110
1.100
1.090
1.080
1.070
1.060
1.050
1.040
1.030
1.020
1.010
1.000
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34

FIGURE 2.--VERO cell adapted virus in fractions 11, 12, and 13 (Figure 1) dialyzed and negatively stained by the pseudoreplica technique. X 175,000.



Size of Purified Virus Particles

The size of VERO cell virus was determined by measuring the greatest diameter of the more spherical particles in the electron micrographs of the results of nine different purification experiments. Measurements were not made of pleomorphic particles. The number of dark particles measured (n_d), 74, had a mean size ($\bar{y}d$) of 93.6 nm. The number of light particles measured (n_l), 31, had a mean size ($\bar{y}_L$) of 113 nm. The sample variance (S'^2) of each type of particle was calculated from (33):

$$S'^2 = \frac{\sum_{n} y_i^2 - \frac{(\sum_{n} y_i)^2}{n}}{n}$$

with the following results: $S'_d{}^2 = 80.8$; $S'_L{}^2 = 53.5$; and standard deviations of 8.99 and 7.31, respectively.

Difference in Size Between the Light and Dark Virus Particles

The light and dark particles which were measured are small samples of the total population of all virus particles observed. The light and dark virus particles differed in size, shape, and electron density. A point estimator (E) of the difference ($\bar{y}_L - \bar{y}_d$) in size (113 - 93.6) of the particles is 19.4 nm. The standard deviation ($\sigma_{(\bar{y}_L - \bar{y}_d)}$) of E is calculated from the variances of each type of particle ($\sigma_L{}^2$ and $\sigma_d{}^2$) as follows:

$$\sigma_{(\bar{y}_L - \bar{y}_d)} = \sqrt{\frac{\sigma_L^2}{n_L} + \frac{\sigma_d^2}{n_d}}$$

The $\sigma_{(\bar{y}_L - \bar{y}_d)}$ of E can be calculated from the sample variances, S'^2_L and S'^2_d, which are approximately equal to σ_L^2 and σ_d^2:

$$\sigma_{(\bar{y}_L - \bar{y}_d)} = \sqrt{\frac{S'^2_L}{n_L} + \frac{S'^2_d}{n_d}} = \sqrt{\frac{53.5}{31} + \frac{80.8}{74}}$$

$$\sigma_{(\bar{y}_L - \bar{y}_d)} = 1.68$$

With a confidence coefficient of 0.95, the error, $2\sigma_{(\bar{y}_L - \bar{y}_d)}$, in estimating the difference between the mean size of each type of particle in the population by the difference in the mean size of each type of particle in the sample is 3.36 nm, according to the formula:

$$2\sigma_{(\bar{y}_L - \bar{y}_d)} = 2(1.68) = 3.36$$

The difference in the mean size between the two types of particles is:

$$(\bar{y}_L - \bar{y}_d) \pm 2\sigma_{(\bar{y}_L - \bar{y}_d)} = 19.4 \pm 3.36 \text{ nm}$$

$$= 16.0 \text{ to } 22.8 \text{ nm}$$

Morphology of Virus in Allantoic Fluid

Ultracentrifugation of virus concentrated by negative pressure dialysis resulted in a white band, comprising

fractions 12 through 14, two-thirds of the linear distance from the top of the tube. These fractions correspond to the peak of maximum radioactivity in VERO cell virus precipitated by PEG and purified by ultracentrifugration. These pooled fractions contained light and dark virus particles (Figure 3) identifical in size and appearance to VERO cell virus which was precipitated by PEG and purified by ultracentrifugation (Figure 2). Projections were not observed on any virus particles.

Normal allantoic fluid, processed as above, did not have a white band and virus particles were not present.

VERO Cell Virus Concentrated by Filtration

Virus directly from the roller bottle cultures contained 10^5 infectious doses 50% per ml. There was a 20 fold concentration by membrane filtration of the cultural medium and a 20 fold increase in viral infectivity. The ratio of light to dark particles was approximately 3:1. Surface projections were absent, and the edges and surfaces of the light particles were smooth and continuous (Figure 4). The size and shape of the particles were identical to virus purified from either VERO cells or allantoic fluid (Figures 2 and 3, respectively).

Morphology of Sectioned Virus

VERO cell and allantoic fluid virus had a three layered unit membrane structure (dense outer rim, transparent

layer and dense innermost layer) around and part of a dense internal component (Figure 5). The intact, round, 108 nm diameter virus photographed was selected from many particles. Only a few of the particles were sectioned through the center and well stained, and many appeared to be disintegrating. Bulbous projections were not observed. These results agree with those of Apostolov et. al. who examined virus pellets from allantoic fluid (4).

Release of VERO Cell Virus

The radioactivity in respective peaks increased from the 30th hour to the 51st hour where the highest peak in extracellular fluid occurred jointly with the maximum CPE (Figure 6). There was a marked decrease at the 58th hour.

After collecting the sucrose gradient fractions, the pellet and centrifuge tube contained the following net counts of radioactivity:

30 hours	46.6 cpm
37 hours	154. cpm
44 hours	152. cpm
51 hours	546. cpm
58 hours	1010. cpm

The specific gravities of the peak of radioactivity in each sample ranged from 1.178 to 1.168.

The counts of ^{3}H uridine recovered by TCA precipitation and NaOH hydrolysis are assumed to be RNA, either

FIGURE 3.--Virus in allantoic fluid which was concentrated by negative pressure dialysis and purified by sucrose density gradient centrifugation at 234,000 X g for two hours at 4^0, dialyzed and negatively stained with 2% PTA. X 146,000.

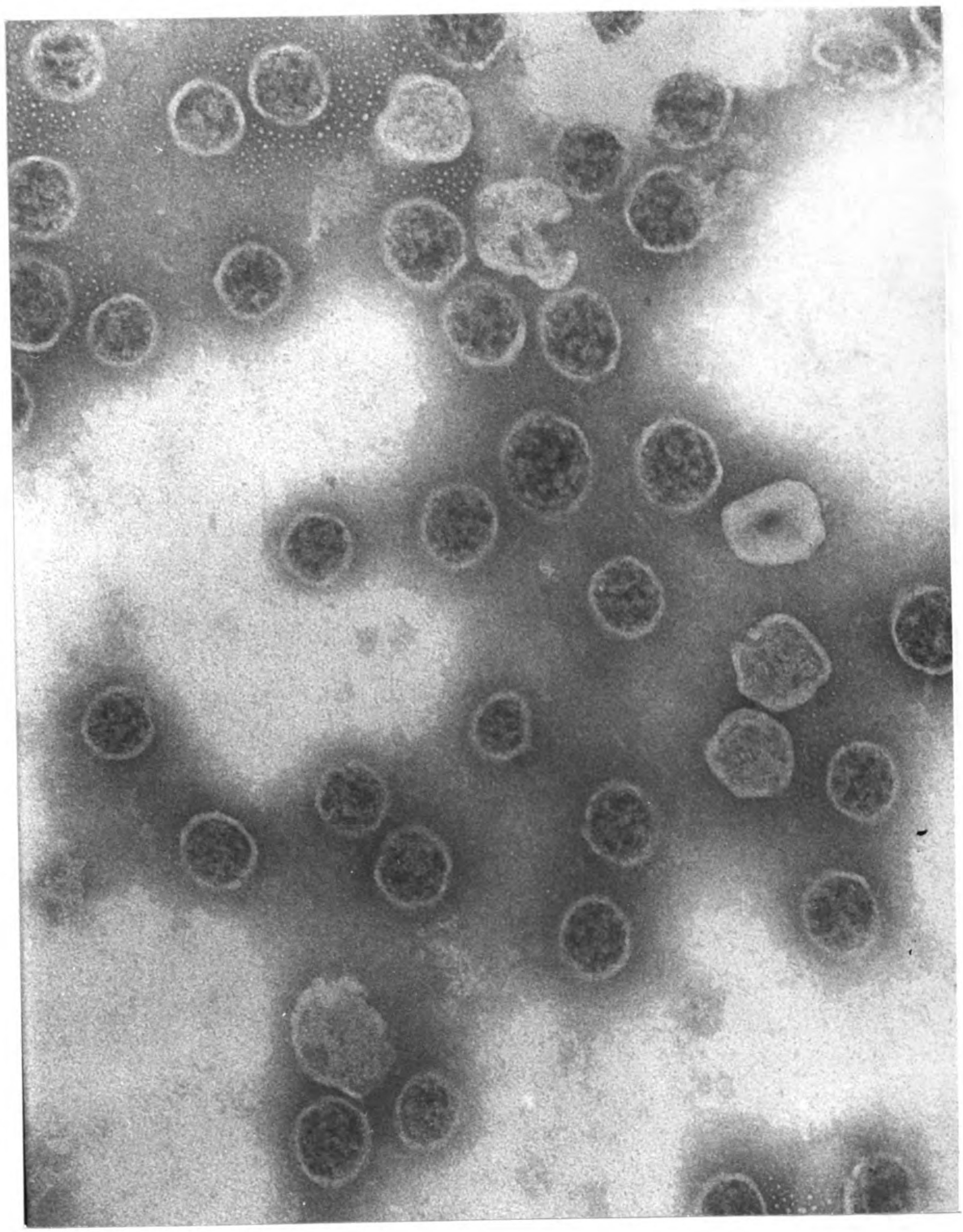


FIGURE 4.--Two light VERO cell adapted virus particles concentrated by filtration and stained with PTA using a nebulizer. X 421,500.

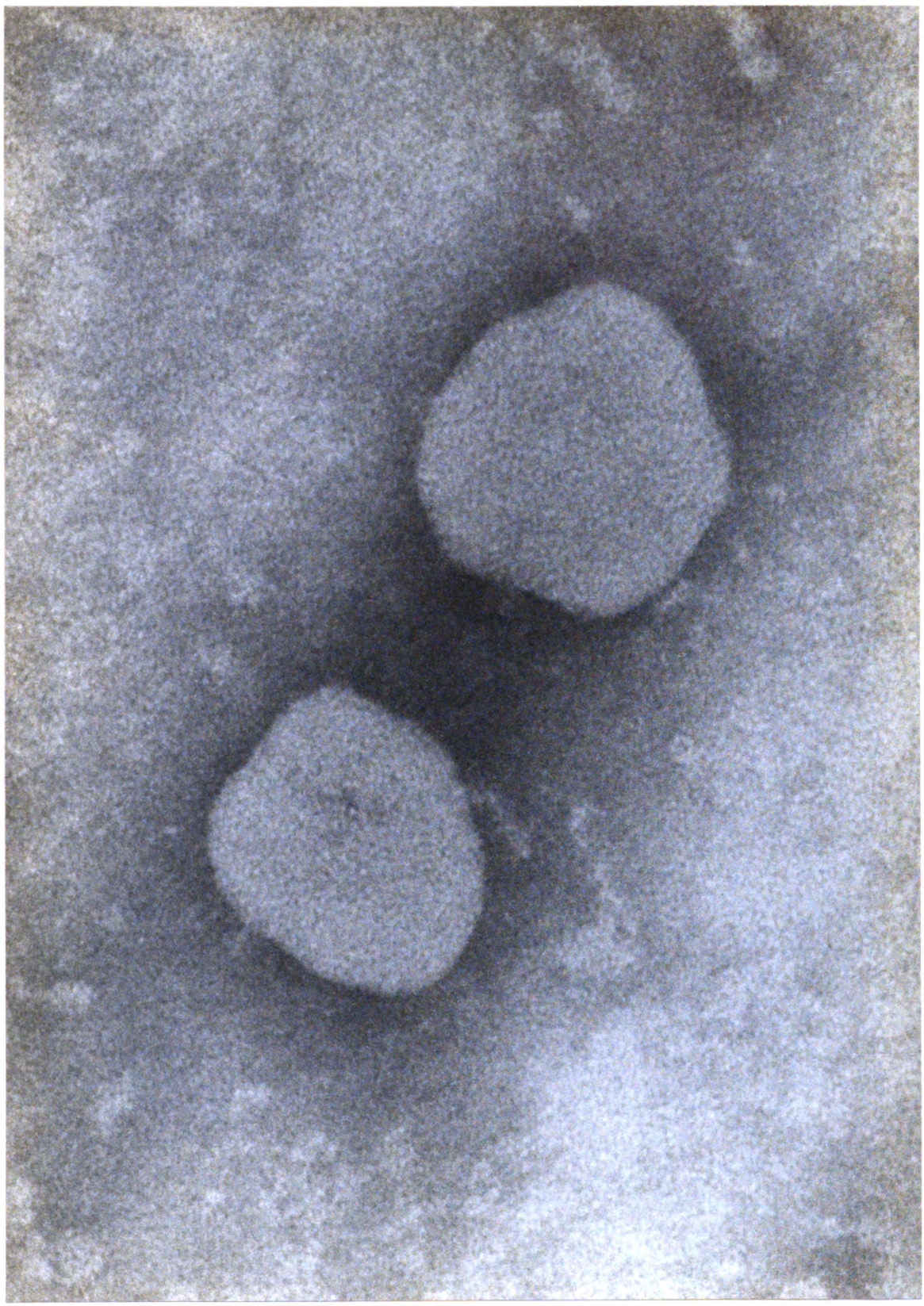


FIGURE 5.--Section of VERO cell adapted virus pelleted by ultracentrifugation, fixed in glutaraldehyde and stained with uranyl acetate and lead citrate. X 335,000.

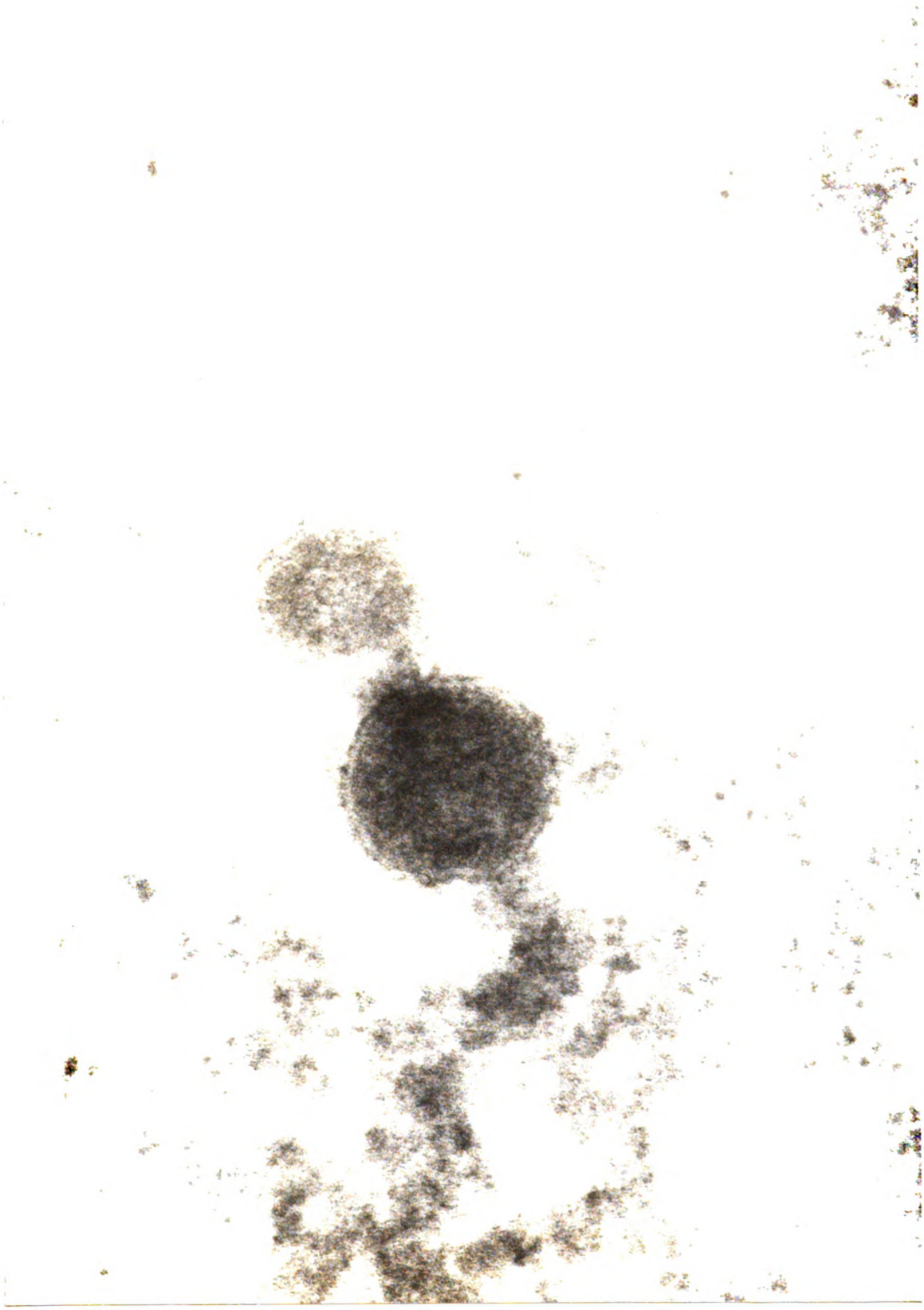
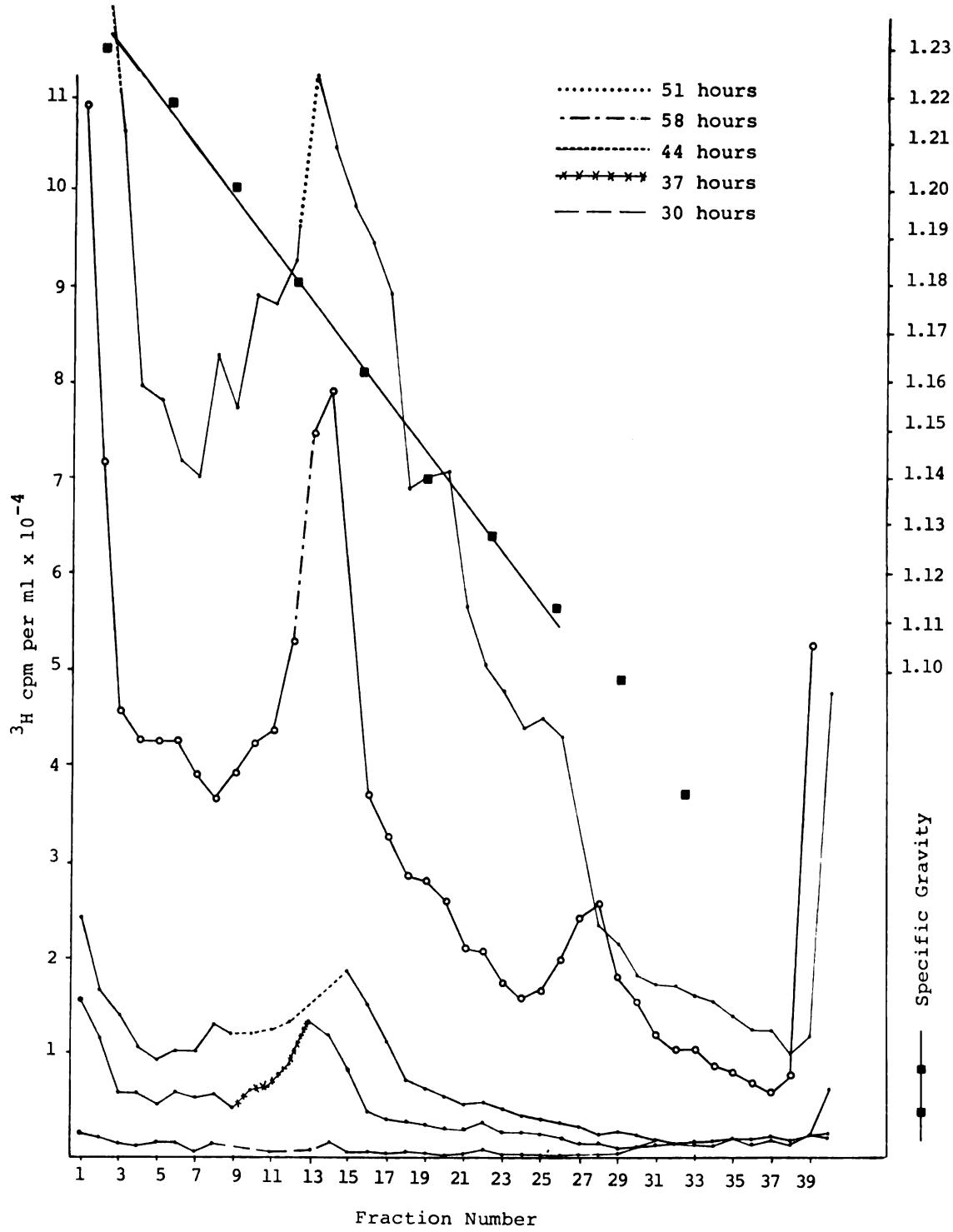


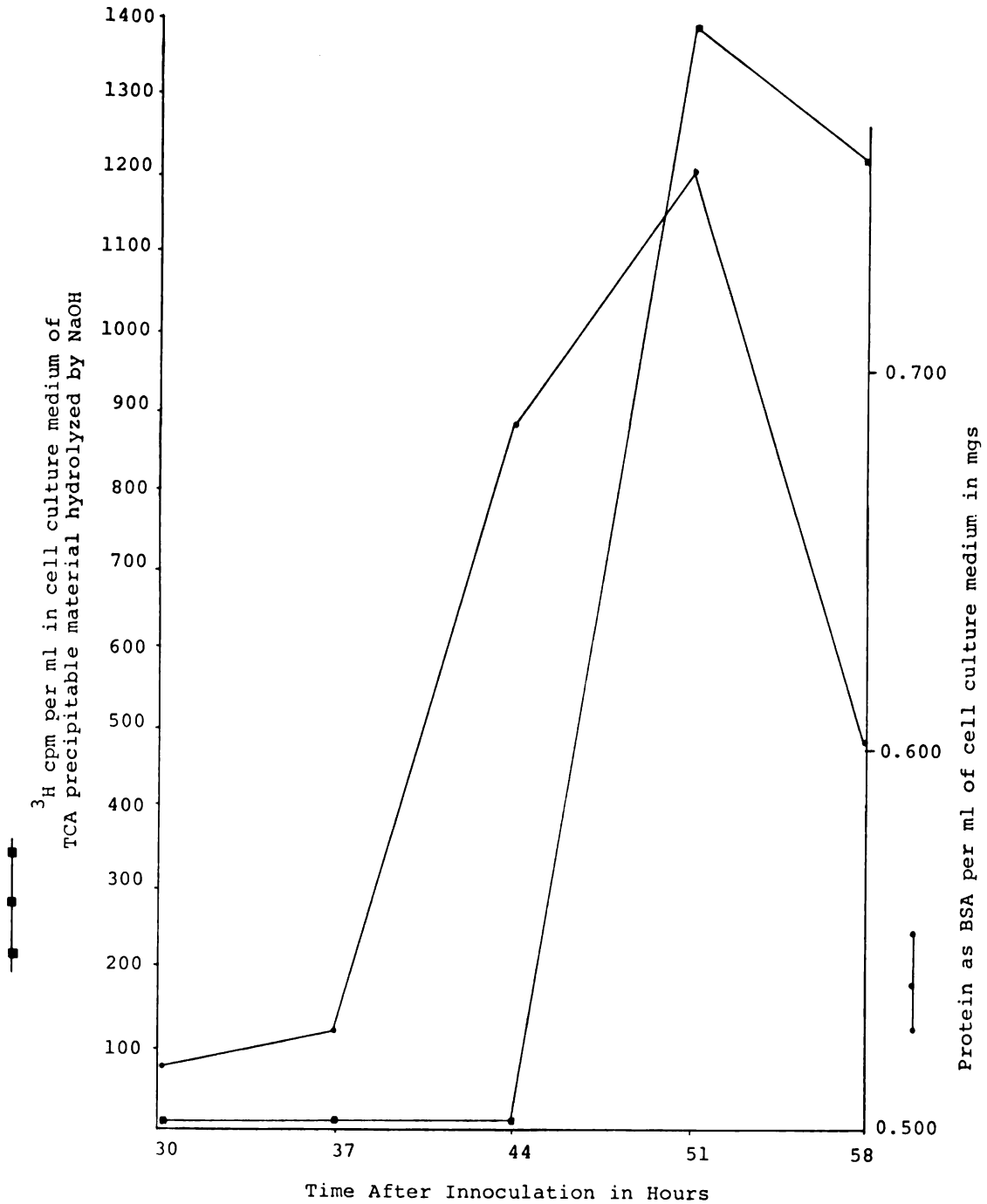
FIGURE 6.--Sucrose density gradient centrifugation of PEG precipitate from virus infected VERO cell culture medium which was collected 30, 37, 44, 51 and 58 hours after inoculation. ^{3}H uridine was added to each sample 12 hours after inoculation.


51 hours
58 hours
44 hours
37 hours
30 hours
^{3}H cpm per ml x 10^{-4}
Specific Gravity
Fraction Number
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39
1 2 3 4 5 6 7 8 9 10 11
1.10 1.11 1.12 1.13 1.14 1.15 1.16 1.17 1.18 1.19 1.20 1.21 1.22 1.23

virus, viral RNA or cellular RNA, released from VERO cells during viral replication (Figure 7). The RNA in the medium increased sharply between 44 and 51 hours and decreased between 51 and 58 hours after inoculation.

Protein as BSA in the culture medium increased sharply between 37 and 51 hours and decreased from 51 to 58 hours after inoculation (Figure 7).

FIGURE 7.--Counts per min of ^{3}H uridine which was precipitated by TCA and hydrolyzed by NaOH and protein as BSA released from VERO cells during viral replication. ^{3}H uridine was added to the cells 12 hours after inoculation.


1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
^{3}H cpm per ml in cell culture medium of TCA precipitable material hydrolyzed by NaOH
0.700
0.600
0.500
Protein as BSA per ml of cell culture medium in mgs
30
37
44
51
58
Time After Innoculation in Hours

DISCUSSION

The primary objective of this study was to successfully concentrate and purify IBV-42 from large volumes of cultural medium from VERO cells in which the virus was propagated and to use this for biochemical and biophysical studies. The most startling observation was that the virus remained infectious after PEG precipitation, concentration by negative pressure dialysis or by filtration, but the same virus was noninfectious after centrifugation at high gravitational force through a sucrose gradient. Precipitation using PEG followed by centrifugation was then studied in detail. Precipitation by PEG was probably due to adsorption or aggregation of virus to PEG molecules which were anionically charged at pH 9. No precipitate was formed in cultural medium which did not contain virus. Before the addition of PEG, virus in the cultural medium was infectious, but during sucrose gradient centrifugation at 234,000 X g for two hours, the virus was rendered noninfectious. Virus in allantoic fluid remained infectious after two cycles of sucrose gradient centrifugation at 109,000 X g by Tevethia et. al. (46). In retrospect, the effect of the high g force was probably physically responsible for many of the unexpected discrepancies encountered later in morphology and infectivity of

the virus. The higher centrifugal force was first used because of limited access to the centrifuge and also on the assumption that the virus could be purified by centrifugation for two hours.

The absence of surface projections, marked differences in physical appearance between the electron light and dark particles, and the ratio between them was not considered of great importance until later when electron micrographs of PEG precipitated and filtered viruses were compared. The ratio of light to dark particles was approximately 1:3 in virus precipitated by PEG followed by centrifugation and 3:1 in those that were only concentrated by filtration. Other than the absence of projections, the light particles were pleomorphic and morphologically identical to IBV-42 described by Berry et. al. (8). The dark particles were uniformly round, morphologically appeared to be identical to negatively stained trysin modified virus (35), and were 16.0 to 22.8 nm smaller than the pleomorphic light particles. These findings suggest that centrifugation caused detachment of the projections from the virus and damaged the virus to the extent that the PTA could penetrate into the interior of the virus and stain the internal components. Apostolov et. al. (4), working with sectioned virus from allantoic fluid, suggest that the projections are extensions of the outer layer of the envelope and that breaks in the envelope, after detachment of the projections, may explain why the

infectivity of this virus is so labile. Becker et. al. (6) were able to demonstrate the projections on negatively stained extracellular virus but not on sectioned virus. Sectioned virus in the present study was identical in appearance to the findings of Becker et. al. (6). Surface projections were not observed even though three different negative staining techniques were used on virus purified by PEG precipitation and centrifugation. While the initial objective was not accomplished with this purification method, it afforded the opportunity to study the internal structure of the virus.

Virus in allantoic fluid, concentrated by negative pressure dialysis and purified by centrifugation at high centrifugal force in a sucrose gradient, had the same appearance as VERO cell virus precipitated by PEG and purified by centrifugation. Virus was noninfectious in both cases after centrifugation.

The absence of surface projections on the virus, which was infectious after membrane filtration, suggests in parallel with the results of other investigators (24) that the projections might not be absolutely essential for cellular infection by IBV-42. The role in infection of the surface projections on IBV and their antigenic nature are major problems for future investigation.

Replication of the virus as measured by radioactivity precipitated by PEG followed by centrifugation in a sucrose

gradient was according to a typical growth curve reaching a maximum at 51 hours and declining at 58 hours. The decline in radioactivity, protein and BSA, and RNA were probably the result of degradation of the virus by cellular enzymes released by lysis of the cells.

The increase in radioactivity of the pellet from the sucrose gradients between 51 and 58 hours after infection was the result of the morphogenesis of the virus. Virus in either VERO cells or the chorioallantoic membrane buds from the cytoplasm into vacuoles which later occupy most of the cytoplasmic space of many cells (6,18). Intracellular virions in various stages of maturation are released when the cells rupture. Membrane bound virions, too small to be removed by clarification centrifugation, probably accumulate in the medium, precipitate with PEG, and sediment in the pellet when centrifuged in sucrose gradients. This would account in the present study for the marked increase in radioactivity from the 44th to the 58th hour after infection.

Even though virus precipitated by PEG and purified by centrifugation was noninfectious, this method is reproducible and allows the replication of IBV-42 in VERO cells to be partially characterized, and the internal structure to be more physically discernible. The sequential steps in the processing of virus to yield dark particles, and the particles themselves, might be an excellent method for investigation of the role of the surface projections as an

integral part of the infectious virion. In addition, the role of the projections as antigenic and immunogenic determinants, attachment of the projections to the virus before centrifugal stress, and possible disruption of the surface of the virus when they are removed, and the internal structure remain enigmas for future investigators.

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