AN ABSTRACT OF THE THESIS OF

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in MICROBIOLOGY (Major) presented on January 24, 1970 (Date)

Title: THE OREGON SOCKEYE SALMON VIRUS: A. BIOPHYSICAL AND BIOCHEMICAL CHARACTERISTICS B. ANTIGENIC RELATIONSHIP TO TWO OTHER SALMONID VIRUSES

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Abstract approved: K. S. Pilcher

The Oregon sockeye salmon virus (OSV) was isolated from diseased sockeye salmon (Oncorhynchus nerka) fingerlings in 1958 by J. L. Fryer. Experimentation performed prior to the research reported herein indicated that the OSV contained essential lipids, was 100 to 300 mμ in size, and possessed RNA (presumptively identified by 5-bromodeoxyuridine treatment of OSV-infected cell cultures).

In the present investigation, OSV was propagated in one of two cell lines derived from the embryonic tissues of either sockeye or chinook (O. tshawytscha) salmon. Infectious cell culture medium was purified by differential centrifugation alone, or in combination with RNase and DNase treatment, and rate-zonal sucrose gradient centrifugation.

OSV suspensions had a propensity for forming viral aggregates during differential centrifugation, presumably caused by pelleting

virions in the presence of host cell debris and serum proteins. Partially purified virus suspensions were fractionated into four visible bands by rate-zonal or isopycnic sucrose gradient centrifugation. Most virus infectivity was detected in two closely associated bands in the middle of the gradient. Evidence suggested that the more dense, faster sedimenting band contained infectious virus and the other band was composed of incomplete, noninfectious virus particles. The sucrose density of the virus band was determined to be 1.16 g/cm^3. The two other visible bands, one above and one below the presumably heterogeneous virus band, were considered to represent nonviral material complexed with varying amounts of virus. Large amounts of virus infectivity were lost either during sucrose gradient centrifugation or when sucrose concentrations were reduced in gradient fractions by dilution and/or dialysis.

Purified suspensions of OSV were treated with hot perchloric acid in order to extract viral nucleic acid. RNA and DNA concentrations in the extracts were estimated with the orcinol and diphenylamine tests. Concentrations of RNA were at least 14 times higher than those of DNA. Viral nucleic acid was also extracted from purified ^{32}P-labeled OSV suspensions by the phenol method at $4^{\circ}C$. Spectral properties of the resulting nucleic acid solutions indicated that they contained relatively large amounts of protein. Viral nucleic acid formed a single band at densities of 1.58 to 1.59 g/cm^3 when subjected to isopycnic cesium sulfate gradient centrifugation.

Rate-zonal glycerol gradient centrifugation of ^{32}P-OSV nucleic acid resulted in the formation of a diffuse band of radioactivity with its peak at 26 S and a pronounced shoulder at 37 S. RNase treatment of aliquots from glycerol gradient fractions reduced all trichloroacetic acid-precipitable ^{32}P-radioactivity by 85 to 97%. Anion exchange chromatography of alkaline-hydrolyzed ^{32}P-viral nucleic acid was used to determine its base composition. Percentage base compositions were cytidylic acid, 25.8 ± 0.6%; adenylic acid, 23.0 ± 0.8%; uridylic acid, 27.7 ± 0.6%; and guanylic acid, 23.4 ± 0.4%. The forementioned experimental data demonstrate that the OSV virion contains single-stranded RNA.

Rabbit-immune sera were produced against OSV, infectious hematopoietic necrosis (IHN) virus, and Sacramento River chinook disease (SRCD) virus. The latter two viruses were isolated from diseased sockeye and chinook salmon, respectively. Differentially centrifuged virus suspensions, containing 5.0×10^8 to 2.0×10^9 $TCID_{50}$/ml, were injected undiluted or emulsified with Freund's adjuvant into rabbits. The antigenic relationship between OSV, IHN virus, and SRCD virus was investigated using cross plaque neutralization tests with each antiserum versus the three viruses. Fifty percent plaque neutralization end points determined in these tests indicated that all three viruses were antigenically related, with OSV and IHN virus being indistinguishable.

Differentially centrifuged, glutaraldehyde-fixed and unfixed

suspensions of OSV were stained with phosphotungstic acid and examined with an electron microscope. The most numerous type of particle in fixed preparations was bullet-shaped with average dimensions of 98 x 166 mμ. However, the most abundant type of particle in unfixed suspensions consisted of two roughly spherical (80 mμ in diameter), closely associated particles. The discrepancy between the appearance of fixed and unfixed OSV suspensions was not experimentally resolved.

The Oregon Sockeye Salmon Virus:
A. Biophysical and Biochemical Characteristics
B. Antigenic Relationship to Two Other Salmonid Viruses

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

June 1970

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ACKNOWLEDGMENTS

I am indebted to Drs. K. S. Pilcher, John L. Fryer, George S. Beaudreau, and Donald E. Mattson for their help and advice during my predoctoral training. Dr. K. S. Pilcher's assistance in the preparation of this manuscript was invaluable.

My deepest gratitude goes to my wife Alice. Without her sacrifices, patience, and understanding, the task of completing my Ph. D. requirements would have been much more difficult. She also played a major role in preparing this thesis, by typing and proof reading.

I also thank my infant daughter Jennifer for her good natured cooperation and the joy she has added to my life.

This research was supported by a Predoctoral Research Fellowship from the Federal Water Pollution Control Administration of the U. S. Department of the Interior (Fellowship number 5F1-WP-26,157-03). I am grateful for their generosity.

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INTRODUCTION

The Pacific salmon, Oncorhynchus species, are of major economic and recreational importance to the United States and Canada. In order to maintain and increase the populations of these fishes, numerous fish hatcheries are operated which hatch and rear young salmon for eventual release into ocean-connected rivers and lakes. As a result of artificial conditions imposed upon them, millions of these hatchery-reared salmon have died during the last 30 years from bacterial, fungal, and viral diseases.

At present, three different viruses, isolated in hatcheries from Pacific salmon, are being investigated in various laboratories. The Sacramento river chinook disease (SRCD) virus was obtained from chinook salmon (O. tshawytscha (Walbaum)) fingerlings in 1965 (Nims, 1968). The Oregon sockeye salmon virus (OSV) and infectious hematopoietic necrosis (IHN) virus were both isolated from sockeye salmon (O. nerka (Walbaum)) fingerlings in 1958 (Fryer, 1964) and 1967 (Amend, 1969), respectively.

In order to comprehensively investigate a newly isolated virus, such as the OSV, it is important to classify it relative to other viruses. If the virus can be classified with a group of known viruses,

then advantage can be taken of knowledge gained by other researchers with similar viruses.

The primary purpose for initiating the research reported in this thesis was to elucidate the biophysical and biochemical properties of the OSV necessary for its classification. Techniques were employed to obtain direct evidence for identification of the virus nucleic acid type, and the size and morphology of the virion.

Another main objective of this thesis was to explore the antigenic relationship between the OSV, IHN virus, and SRCD virus. Rabbit-immune serum was developed against each of the viruses, and serum virus-neutralization tests were used to investigate antigenic similarities.

This research program was begun in June, 1966 and was finished in September, 1969.

LITERATURE REVIEW

Since the early 1950's, three virus-like agents have been isolated from different epizootics among sockeye salmon, Oncorhynchus nerka, in the Pacific Northwest. The first isolate, obtained in north-central Washington state, was described by Rucker et al. (1953). They found the infectious agent to be filterable and resistant to chemotherapeutic compounds. Watson, Guenther, and Rucker (1954) further characterized these disease-producing filtrates and investigated the source and transmission of the disease. Succeeding experimentation was confined to epizootiology, histopathology, and hematology of diseased sockeye salmon (Wood and Yasutake, 1956; Watson, Guenther and Royce, 1956). Unfortunately, the probable virus isolate was lost in storage, and since the original epizootic had been eliminated, it could not be recovered. A more extensive review of this agent was given by Parisot, Yasutake and Klontz (1965).

The original isolate was first named sockeye salmon virus, but later it was renamed the Columbia River sockeye disease (CRSD) virus (Parisot et al., 1965) to differentiate it from another similar agent isolated in Oregon by J. L. Fryer in 1958 (Fryer, 1964). After determining that the infectious entity was filterable and specific for sockeye salmon, Fryer undertook a

research program to develop techniques for initiating and maintaining cell lines derived from embryonic tissues of salmonids (Fryer, Yusha, and Pilcher; 1965; Fryer, 1964). Using a sockeye salmon cell line, Wingfield, Fryer and Pilcher (1969) were able to propagate the agent in vitro. Medium from infected cell cultures produced typical disease symptoms in fingerling sockeye salmon, and filtrates from the diseased fish caused characteristic cytopathic effects (CPE) in cell cultures (Wingfield, 1968). This virus has been referred to as the Oregon sockeye salmon virus (OSV) (Wingfield, 1968), the Oregon sockeye disease (OSD) virus (Parisot et al., 1965), and the sockeye salmon virus (Oregon strain) (Wingfield et al., 1969). The term Oregon sockeye salmon virus (OSV) will be used in the remainder of this thesis.

Most recently, in 1967, a virus named infectious hematopoietic necrosis (IHN) virus was isolated from sockeye salmon in Cultus Lake, British Columbia, Canada (Amend, 1969). Serial passages of this agent in the fathead minnow cell line (FHM) Gravell and Malsberger, 1965) were performed. Cell culture medium containing virus was injected into juvenile sockeye salmon, and virus was recovered from moribund fish (Amend, 1969).

Research with the Oregon sockeye salmon and IHN viruses has emphasized determination of taxonomically significant properties necessary for virus classification. Although there is

no universally accepted scheme for classifying animal viruses, the following chemical and morphological properties of viruses have been accepted for differentiating major groups: (1) type and properties of nucleic acid, (2) sensitivity to ether, (3) symmetry of the nucleocapsid, and (4) size and shape of the virion or nucleocapsid (Andrewes, 1968; Lwoff, Horne and Tournier, 1962; Wilner, 1969).

Animal viruses are divided into two main groups by the type of nucleic acid they contain. Among RNA viruses, the number of strands in the RNA molecule of the mature virus is sometimes used to further subdivide this group (Andrewes, 1968; Bellett, 1967).

Another important chemical property of viruses is the presence of virus-associated lipids. This characteristic is most commonly determined by exposing virus particles to ether or chloroform; sensitive viruses contain essential lipids and their infectivity is destroyed by ether; resistant ones do not contain such lipids. In some cases, viruses which are released from infected cells by budding through cell membranes possess envelopes containing lipoproteins.

The presence of an envelope (determined by electron microscope observation) is one of several morphological characteristics that are used to classify viruses. The structural symmetry of the nucleocapsid is commonly used to separate the RNA and DNA

viruses into major subdivisions. Types of symmetry include helical, icosahedral or cubic, and complex. With icosahedral viruses, the number of capsomeres represents an important characteristic. Lastly, the size and shape of the virion or nucleocapsid are given varying degrees of value by viral taxonomists.

In the classification of animal viruses, the above chemical and structural properties are sufficient for placing viruses into main groups, such as the Myxovirus and Adenovirus groups. However, differentiating the subgroups and strains within these groups requires additional criteria.

These criteria are of two general types, those related to the biochemical and biophysical nature of the virus, and those concerned with characteristics of in vivo and in vitro virus propagation. Antigenic relationships among viruses have been used extensively to subdivide some groups. Other properties of a virus commonly used as criteria are its stability to pH and heat; effects of chemical agents, such as trypsin or formalin (Andrewes, 1968); and the ability to cause agglutination of the erythrocytes of certain animal species.

In vitro (tissue culture) systems are used to delineate the following aspects of virus infection: (1) formation of intracellular inclusions, (2) intracellular site(s) of virus development, (3) cytopathic effects, and (4) specificity for various host cells.

The mode of transmission, host specificity, symptomatology, and pathology of virus infections in vivo were quite important in earlier animal virus classification systems. However, more recently, taxonomists have placed less emphasis on these characteristics.

Some of the properties mentioned above have been determined for the Oregon sockeye salmon virus. The type of viral nucleic acid was presumptively identified as RNA by Wingfield et al. (1969) using 5-bromodeoxyuridine (BUDR) treatment of virus-infected cell monolayers. BUDR had no effect on the replication of the OSV in these cell cultures, while a known DNA virus-cell system treated similarly showed no detectable release of new virus. Wingfield et al. also found OSV to be inactivated by exposure to 20 percent ether, which strongly indicates the presence of virus-associated essential lipids.

Both Wingfield et al. (1969) and Parisot et al. (1965) used Millipore membrane filtration to estimate the size of the OSV particle. The former authors reported a particle size estimate of 110 to 165 mμ, and the latter estimated a diameter greater than 100 mμ and less than 300 mμ.

Amend and Chambers (1969) examined negatively stained preparations of OSV with an electron microscope. In "monolayer tissue culture concentrates," they observed bullet-shaped

particles with an average width of 91 mμ and a mean length of 181 mμ. No well defined envelope was seen. These authors tentatively placed the OSV in the Rhabdovirus group.

Fenner (1968) describes the structure of Rhabdoviruses as bullet-shaped, 70 x 175 mμ in size, with an envelope. They contain single-stranded RNA, and the nucleocapsid has helical symmetry. Examples of viruses placed in this group are vesicular stomatitis virus (VSV) and rabies virus.

Additional characteristics of the OSV have been elucidated and may be helpful in distinguishing it from related viruses. Wingfield (1968) performed experiments to determine in vitro and in vivo host specificity. In vitro, autonomous cell lines derived from chinook salmon and steelhead trout embryonic tissue both supported virus development equally well and showed typical cytopathic effects when exposed to OSV for the first time. However, a similarly derived established cell line from coho salmon (Oncorhynchus kisutch (Walbaum)) and a primary chinook cell line were apparently unaffected by inoculation with OSV.

In his in vivo experiments, Wingfield (1968) observed the OSV to be very host specific. He inoculated OSV into the water of several containers which held either kokanee (O. nerka), chinook, or coho salmon, or rainbow trout fingerlings. Only the kokanee salmon fingerlings had significant mortalities.

Nims (1968) investigated the specificity of the OSV for two of the salmonid cell lines used by Wingfield (1968) in his in vitro experiments. She performed five serial passages of OSV in the chinook salmon (CHSE-114) and the steelhead trout (STE-137) cell lines. The increase of infective units ($TCID_{50}$) and the appearance of CPE during passage through STE-137 were similar to the increase and CPE observed in the sockeye salmon cell line routinely used for virus propagation. However, the first passage of OSV in cell line CHSE-114 resulted in only a slight increase in $TCID_{50}$ units and no CPE was observed. Subsequent serial passages in CHSE-114 yielded greater increases in infectivity and the appearance of typical CPE, but the ultimate level of virus propagation was about ten times less than that observed in the other two cell lines. Apparently, either cell line CHSE-114 or the stock of OSV had changed during the period between the experiments of Wingfield (1968) and Nims (1968).

Both Nims (1968) and Wingfield (1968) used phase microscopy to examine the CPE of OSV in salmonid cell lines. In a sockeye salmon embryonic line, Wingfield observed thickened nuclear membranes and abnormally dense nucleoli 24 hours after infection. After 48 hours, infected cells were beginning to round up, and after 96 hours many more rounded cells were seen; at both times, nuclei in rounded cells showed abnormalities seen at

24 hours. Nims observed similar CPE in the CHSE-114 and STE-137 cell lines infected with OSV.

Wingfield (1968) also stained OSV-infected sockeye salmon cell cultures with May Grunwald-Giemsa stain. Early effects of virus infection were also seen associated with cell nuclei. Instead of nucleoli becoming more optically dense as seen with the phase microscope, the nucleoli disappeared, nuclear membranes became more thick and nuclear chromatin became denser and more fibrous.

Another distinguishing characteristic of OSV is the temperature range in which virus replication occurs *in vitro*. Wingfield (1968) and Wingfield *et al.* (1969) were able to measure virus production in infected sockeye salmon embryonic cell cultures from 4 to 20° C. At 23° C, however, no increase in virus concentration or CPE was detected. Optimum virus replication *in vitro* was observed between 13 and 18° C.

METHODS AND MATERIALS

Cell Lines

Cell lines SSE-30 and CHSE-214 were used for propagation of the OSV, SRCD virus and IHN virus. Both lines were initiated from embryonic tissues of salmon by the method of Fryer et al. (1965). SSE-30 was started in October, 1965 and CHSE-214 in October, 1964. At the time of this writing, CHSE-214 has been transferred 80 times, and SSE-30 has had approximately 60 transfers.

Maintenance of Stock Cell Cultures

Stock cultures were grown in 32-oz prescription bottles sealed with latex stoppers (West Co.). Cultures were transferred at 20 to 30 day intervals using a method described by Fryer et al. (1965) with the following modifications: cell monolayers were removed with 0.2% trypsin (Difco) in GKN solution (Merchant, Kahn and Murphy; 1964); growth medium was complete Eagles Minimal Essential Medium (MEM) (Eagle, 1959); and cell suspensions from cell lines SSE-30 and CHSE-214 were seeded at 6×10^5 and 4×10^5 cells per ml, respectively. Three types of MEM were used: Earle's Balanced Salt Solution (BSS) (Earle, 1943)

with sterile MEM components (Microbiological Associates); powdered MEM (GIBCO) sterilized by Millipore filtration; and autoclavable MEM (Auto-Pow, Flow Laboratories). Growth medium will be referred to as complete MEM in the remainder of this thesis. Complete MEM contained 10% agamma calf serum (Hyland Laboratories), penicillin (100 units/ml), streptomycin (100 µg/ml), and mycostatin (25 units/ml). Growth medium on the stock cultures was changed every eight to ten days.

Each time cell cultures were transferred, the cell suspensions were checked for sterility by inoculating one ml of fluid into tubes containing 10 ml of either Thioglycollate broth (Difco) or M-13 Mycoplasma broth (Stevens, 1969). Sterility checks were incubated at 35°C for two weeks.

Viruses

Oregon sockeye salmon virus (OSV)

A homogenate of kidneys from diseased sockeye salmon fingerlings was prepared by J. L. Fryer in 1958. The homogenate was mixed with sterile skim milk, lyophilized and stored at -60°C. The Oregon sockeye salmon virus obtained from this lyophilized material was serially passed in sockeye salmon cell cultures; and sterile medium from infected cultures was frozen at -60°C in

sterile Kahn tubes with latex stoppers (Nims, 1968).

The preparation of stock OSV has been described elsewhere (Wingfield et al., 1969). Stock virus used in the following experiments contained 3.0×10^6 plaque forming units (pfu)/ml (assayed after freezing at -60°C) and was mycoplasma-free as determined by sterility checks in M-13 mycoplasma broth (Stevens, 1969).

Sacramento River chinook disease virus (SRCD)

The SRCD virus was first isolated in California from diseased chinook salmon (Oncorhynchus tshawytscha (Walbaum)) fingerlings in 1965. The virus used in this research was originally obtained by Nims (1968) from a California Fish and Game Department laboratory. Stock virus was prepared by Nims in cell line CHSE-214; complete MEM, containing virus, was dispensed into screw-cap tubes and frozen at -60°C. Thawed stock virus had 3×10^7 pfu/ml.

This stock was used to produce stock virus for experiments described in this thesis. Cell lines SSE-30 and CHSE-214 were infected by allowing a 1/100 dilution of Nims' stock to adsorb to the cell monolayer of each culture for two hours at 18°C. The inocula were removed and complete MEM was added to each culture bottle. After incubation at 18°C for three days, the infectious culture medium was centrifuged at 2, 200 x g for 30 min and

dispensed in small volumes into pyrex tubes. The tubes were sealed with latex stoppers and frozen at -60°C. Thawed stock SRCD virus propagated in SSE-30 and CHSE-214 contained 1.8×10^7 and 3.5×10^7 pfu/ml, respectively.

Infectious hematopoietic necrosis virus (IHN)

An isolate of IHN virus was received from the Western Fish Disease Laboratory in June, 1969. The virus was contained in complete MEM from infected FHM cells, and had been frozen in liquid nitrogen in sealed ampoules before shipment. Stock virus, with 8.0×10^6 pfu/ml after freezing at -60°C and thawing, was prepared in SSE-30 cell cultures in the same manner as for the SRCD virus.

Virus Infectivity Assays

The amount of infectivity in suspensions of SRCD, IHN, or Oregon sockeye salmon virus was determined by either the end-point dilution or monolayer plaque assay method. The latter method was used in cross-plaque-neutralization tests, and for assaying the pfu/ml in stock virus suspensions. All other infectivity determinations described in this writing were performed with the end-point dilution method.

End-point dilution method

The end-point dilution technique used in these experiments has been described by Wingfield et al. (1969). Slight modifications were made, however. The cell concentrations used to seed the serological tubes were 6×10^5 cells per ml for cell line SSE-30 and 4×10^5 cells per ml for line CHSE-214. The tubes were used two to five days after seeding.

Monolayer-plaque assay technique

Plaque assays were performed in two-ounce prescription bottles. Eight to ten days before tests were performed, bottles were planted with five ml of cells at concentrations of 1×10^6 and 5×10^5 cells per ml for SSE-30 and CHSE-214, respectively. The cell cultures were incubated at 18°C, and complete MEM was changed once after 4 or 5 days.

To perform an assay, bottles with confluent monolayers were first rinsed twice with three ml of MEM. Virus samples were diluted in complete MEM with 5% agamma calf serum or in MEM containing 0.4% BSA. Assays of stock virus were performed using 5-fold dilutions. For cross-plaque neutralization tests, the virus dilution containing approximately 100 pfu/0.15 ml was used. In each case, three bottles of cells were inoculated for each virus or serum dilution, using a volume of 0.3 ml per bottle. After

adding virus, bottles were tilted every 15 min to distribute the virus which was allowed to adsorb at 18°C for two hours. After adsorption, the bottles were cooled at 4°C for 30 min.

An agar overlay mixture was prepared in one of two ways. Either 2X complete MEM with 10% agamma calf serum was heated to 45°C and combined with an equal volume of sterile, melted 1.5% Ionagar No. 2 (Oxoid) in double distilled water cooled to 45°C; or a 0.8% suspension of Ionagar No. 2 in MEM (Auto-Pow) was autoclaved, cooled to 45°C, and mixed with prewarmed serum, glutamine, penicillin, and streptomycin to give concentrations of 5%, 2.0 mM, 100 units/ml, and 100 μg/ml, respectively. Five ml of agar overlay was distributed over the monolayer in each cooled 2-ounce prescription bottle and the agar allowed to solidify for 1 hour. The bottles were then inverted and incubated at 18°C.

When virus plaques were developed, after about four days of incubation, the bottles were cooled at 4°C for 30 minutes. Three ml of an agar overlay mixture prepared as described above, but without serum and containing 50 μg/ml of neutral red, was layered on top of the original agar sheet in each bottle. The bottles were inverted and incubated in the dark at 18°C (McCombs et al., 1966). Plaques were counted one to two days later. The pfu per bottle for a set of three bottles containing approximately 20 to 200 plaques each was averaged and the pfu per ml calculated.

Virus Propagation

Monolayers of cell lines SSE-30 or CHSE-214 growing in 32-ounce prescription bottles, prepared in the same manner described for stock cultures, were infected with virus 10 to 15 days after being seeded. Two methods were used for virus infection. One involved diluting stock OSV in complete MEM, with 5% agamma calf serum to give a final concentration of 1.5×10^3 $TCID_{50}$/ml. The complete MEM in the bottles was replaced with the 30 ml of complete MEM containing virus. The other method consisted of adsorbing three ml of a 1/100 dilution of stock virus to the cells in each bottle for 2 hours at 18°C. The approximate pfu/cell ratios in this latter procedure were 0.003, 0.005, and 0.03 for OSV, IHN, and SRCD virus, respectively. After adsorption, the inoculum was removed, and 25 ml of complete MEM containing 0.4% bovine serum albumin (BSA) (Fraction V, Nutritional Biochemical Company) instead of serum was added to each bottle.

Infected cell cultures were incubated at 18°C for 72 to 96 hours, until 70 to 90% of the cells had the rounded-up appearance typical of viral CPE (see Literature Review). The medium was then collected and pooled.

Virus Purification

The principal virus purification procedures used in the experiments described in this thesis included differential centrifugation, RNase and DNase treatment, and rate-zonal sucrose gradient centrifugation. The basic steps, materials and equipment used in these techniques are discussed in this section. Additional details will be elaborated upon in succeeding sections dealing with each type of experiment.

Differential centrifugation

Pooled medium harvested from infected cell cultures was dispensed into sterile one-by-three inch polycarbonate tubes (Beckman Instruments, Inc.), and centrifuged in a No. 30 rotor (Beckman Instruments, Inc.) in either a Model L or L2 ultracentrifuge. Depending upon the type of experiment being performed, the tubes were centrifuged at 2,200 x g for 30 min., 8,700 x g for 15 min., or at 19,600 x g for 15 min. (Centrifugal force values are for the midpoint of the long axis of the centrifuge tube.)

The supernatants from the "low-speed" centrifugation were pooled, dispensed into one-by-three inch polycarbonate tubes, and centrifuged at 55,000 x g for 60 min. in a No. 30 rotor. The resulting pellets were loosened and mixed with a small glass stirring rod, then were suspended in approximately 2 ml of one of the

following solutions: MEM; 5% sucrose with 0.005 M ethylenediamine tetraacetic acid (EDTA); or 0.05M Tris buffer with 0.001M EDTA, pH 7.5 (TBE-buffer); or equal volumes of RNase and DNase in Tris buffer (see Nuclease Treatment).

An alternate method of concentrating the virus in the pooled low-speed supernates was used in an effort to eliminate as much cellular material as possible before pelleting the virus. This method employed six-ml, 95% glycerol "pads" in one-by-three inch nitrocellulose centrifuge tubes (Beckman Instruments, Inc.). A 25-ml volume of the pooled supernatant was carefully layered into each of three tubes containing glycerol pads. The tubes were centrifuged at 42,000 x g for 60 min. in an SW 25.1 rotor with the brake on. After each centrifugation cycle, the fluid above the pads was removed without disturbing the glycerol. When all of the sedimentable material had been collected, the layer on each pad was removed with a tube slicer (Beckman Instruments, Inc.). The layers from three tubes were pooled and diluted to 25 ml with TBE-buffer and 95% glycerol to give a final concentration of approximately 40% glycerol. This volume was layered onto another six-ml, 95% glycerol pad, and centrifuged at 42,000 x g for 120 minutes with the brake on. After removing the supernatant and the layer on the pad, the layer was diluted to 25 ml with the TBE-buffer; and the virus was pelleted at 55,000 x g for 60 min. The pellet was

suspended in 2.0 ml of TBE-buffer by the same procedure given in the preceding paragraph.

Nuclease treatment

In experiments where purified virus was to be used for nucleic acid extraction, pellets resulting from differential centrifugation were suspended in a solution containing RNase and DNase. RNase (bovine pancreas, 10X crystallized, Sigma Chemicals) and DNase (beef pancreas, noncrystallized, Sigma Chemicals) solutions were prepared independently at a concentration of 60 µg per ml in 0.05 M Tris buffer, pH 7.0, with 0.001M $MgCl_2$. The solutions were stored at -60°C in one-ml quantities.

One-ml volumes of each enzyme solution were combined and used to suspend the pellets, giving a final RNase and DNase concentration of 30 µg per ml. The suspension was incubated at 25°C for 60 minutes; then it was layered onto a sucrose gradient for further purification.

Rate-zonal sucrose gradient centrifugation

Concentrated virus suspensions (usually after the nuclease treatment) with a volume of approximately two ml were layered on either a 10 to 60% or a 10 to 55% (W/V) sucrose gradient prepared in one-by-three inch nitrocellulose centrifuge tubes. The

gradients were centrifuged in an SW 25.1 rotor at 42,000 x g for 120 min. with the brake off.

Solutions of 10, 55, and 60 percent sucrose were prepared in TBE-buffer. Ten to 55% gradients were produced with a Buchler gradient maker (Buchler Instruments, Inc.). Gradients of 10 to 60% sucrose were prepared in the same manner, except a two-ml 60% sucrose pad was used. The final volume of the gradients was 30 ml. Visible bands formed in the gradient were removed by piercing the bottom of the centrifuge tube and collecting one- or two-ml fractions, or with a tube slicer.

In experiments designed to measure the density of the Oregon sockeye virus, the location of the virus band was established by infectivity assays (see Determination of Virus Density). However, in experiments which involved characterizing nucleic acid extracted from purified unlabeled or ^{32}P-labeled virus suspensions, the duration of a purification procedure was critical due to the instability of virus infectivity or the two-week half life of ^{32} Phosphorous. Since infectivity assays took five to seven days for completion, two other methods for locating the virus band were used.

Each method was dependent upon the previously determined behavior of a virus band in a sucrose gradient centrifuged at 42,000 x g for two hours. The approximate distance of the virus band from the bottom of the centrifuge tube (about 40 mm) was

used to identify an unlabeled virus band. To recover this band, the exact distance in millimeters was determined, and this value was converted to volume using the conversion factor 1 mm=0.46 ml of sucrose, i.e., 34 ml/75 mm. Two-ml fractions were collected and the three fractions corresponding to approximately three ml above and below the measured volume of the band were pooled. Also, the band identified by the above procedure was removed with a tube slicer.

Another behavioral characteristic of a virus band centrifuged under the conditions described above was its migration to a region of the sucrose gradient having a density of approximately 1.14 g/cm^3. The radioactivity and density of one-ml fractions from a gradient containing ^{32}P-labeled virus were measured. The six fractions containing the peak of radioactivity corresponding to a sucrose density of 1.14 g/cm^3 represented the virus band.

Estimation of Virus Purity

At each major step used in the virus purification methods, the virus fraction was tested for infectivity and protein content. Infectivity was determined by the end-point dilution method (Wingfield et al., 1969). Protein concentration was measured according to the method of Lowry (1951) as modified by Shaw and Ingraham (1967).

The values for total $TCID_{50}$'s and μg protein for each step were converted to the ratio of $TCID_{50}$/μg protein. These ratios were then used to estimate the efficiency of each step and the degree of virus purity obtained in a purification procedure.

Another means of estimating the purity of a virus suspension was electron microscopic examination. Suspensions were stained with phosphotungstic acid (PTA) and examined with a Philips EM300 electron microscope. (See section on Electron Microscopy.)

Determination of Virus Density

For Oregon sockeye salmon virus density determinations, cell monolayers of cell line SSE-30 were infected with 1.5×10^3 $TCID_{50}$ units in 30 ml of complete MEM containing 5% agamma calf serum. After incubation for 3 to 5 days, the virus-containing medium was centrifuged at 2, 200 x g for 30 minutes, and the virus was pelleted at 55, 000 x g for 60 minutes. The pellets were suspended in approximately two ml of 0.05 M Tris buffer, pH 7.0. (See Virus Purification)

The virus suspension was layered on a 10 to 60% sucrose gradient which was centrifuged at 42, 000 x g for 16 hours. Fractions of either 110 (1.7 ml) or 120 (2.0 ml) drops were collected through a hole pierced in the bottom of the centrifuge tube. Aliquots were removed from each fraction for infectivity and density

determinations.

Infectivity was assayed with the end-point dilution method. Density was determined by measuring the refractive index of each fraction with a Bausch and Lomb refractometer and converting these values to sucrose density with a conversion table in The Handbook of Chemistry and Physics (1961). The density of the virus was determined by graphically plotting infectivity and density versus fraction number. The sucrose density value corresponding to the center of the peak with greatest virus infectivity was considered the density of the virus.

Characterization of Viral Nucleic Acid

The type of nucleic acid contained in purified suspensions of OSV was determined using both unlabeled and ^{32}P-labeled virus. Extracted nucleic acid from unlabeled virus was identified by spectrophotometric analysis. The nucleic acid from ^{32}P-labeled virus was identified and characterized by its behavior in cesium sulfate and glycerol gradients, by its sensitivity to alkaline hydrolysis and RNase, and by its nucleotide composition determined by anion exchange chromatography.

Unlabeled virus

Virus purification. Monolayers of cell line SSE-30 were infected by replacing growth medium with 30 ml of complete MEM

containing 0.4% BSA instead of serum and 1.5×10^3 $TCID_{50}$/ml of stock virus. When 70 to 90% of the cells showed CPE, the medium was harvested and pooled. The virus was partially purified by differential centrifugation, with a low-speed cycle of 2, 220 x g for 30 min., and with a high-speed cycle of 55, 000 x g for 60 min. One ml each of 60 µg/ml RNase and DNase in Tris buffer (see Nuclease Treatment) were used to suspend the virus pellet in each 30-ml tube. After incubation at 25°C for one hour, the virus suspension was layered on a 10 to 60% sucrose gradient and centrifuged at 42, 000 x g for two hours. Two-ml fractions were collected through the bottom of the centrifuge tube.

The fractions containing the virus band were estimated by measuring the distance of the visible band (shown in previous experiments to possess the greatest infectivity) from the bottom of the tube and converting the distance to volume (see Virus Purification, Rate-zonal-sucrose-centrifugation). Appropriate fractions were pooled, diluted to 50 ml with TBE-buffer, and the virus pelleted by centrifugation at 55, 000 x g for 60 min. The pellet was resuspended and diluted to 25 ml with TBE-buffer and dialyzed against one liter of TBE-buffer for 25 hrs at 4°C with buffer changes at eight and 21 hrs. Elimination of detectable sucrose in the dialysate was assayed by the Orcinol test (Ceriotti, 1955) using absorbance at 550 mµ with a Beckman

Model B Spectrophotometer.

Perchloric acid extraction. Following dialysis, the virus suspension was centrifuged at 55, 000 x g for 60 min, and the pellet suspended in 2. 0 ml of TBE-buffer. A 0. 5 ml aliquot was removed for infectivity titration, and the remaining volume was diluted with an equal volume of 1. ON perchloric acid (PCA). The suspension was incubated at 70°C for 60 min; then it was cooled and centrifuged at 500 x g for 20 min. The supernate was removed, the pellet re-extracted with 1. 0 ml of 0. 5N PCA, and the two extracts were combined (Darlington and Randall, 1963).

Spectrophotometry. The concentration and purity of nucleic acids in the PCA extracts were determined by measuring absorbance in the ultraviolet (UV) region. All measurements were performed in quartz cuvettes (Beckman Instruments, Inc.) and a Gilford spectrophotometer (Model 2000, Gilford Instrument Laboratories, Inc.). To determine nucleic acid concentration, a standard curve was prepared using absorbance of known concentrations of yeast RNA in 0. 5N PCA at 260 mμ. The absorbance of PCA extracts containing viral nucleic acid was measured at 220, 230, 240, 250, 260, 270, 280 and 290 mμ. From the resulting values, the concentration of viral nucleic acid was estimated, and a UV absorption spectrum was obtained.

After the absorbance at UV wavelengths was examined, the

PCA extract was divided into two equal volumes. One volume was tested with the orcinol reaction (Ceriotti, 1955) for the presence of RNA (ribose), and the other was assayed for the presence of DNA (deoxyribose) with the diphenylamine reaction (Burton, 1956).

The orcinol test was performed using simultaneous determinations of RNA and sucrose (Willard, Merritt and Dean; 1965) because residual amounts of sucrose (2 to 5 µg/ml) not removed by dialysis were present in the PCA extracts and interfered with the orcinol reaction (Brown, 1946). The procedures for the test were slightly modified from those described by Ceriotti (1955). A stock solution of 1.25% orcinol (Nutritional Biochemicals Co.) was stored at $4^{o}C$ (Merchant, Kahn and Murphy; 1964), and was used to make fresh orcinol-reaction mixtures for each test. Standard curves for sucrose and RNA were prepared independently using concentrations of 2 to 10 µg/ml yeast RNA (Nutritional Biochemicals Co.) and 2.5 to 7.5 µg/ml sucrose in 0.5N PCA at wavelengths of 550 and 675 µg.

DNA determinations were made according to Burton's modification of the diphenylamine test (Burton, 1956). Standard curves were prepared with deoxyribonucleic acid (Nutritional Biochemicals Co.).

^{32}P-labeled virus

Virus-labeling procedures. Cell lines SSE-30 and CHSE-214 were both used for virus propagation and were treated similarly. Cell monolayers in 32-oz prescription bottles were rinsed twice with 25-ml volumes of MEM. Stock OSV was diluted 1/100 with MEM and adsorbed to the monolayers as described above (see Virus Propagation). After removing the virus inocula, the cell cultures were rinsed once with 25 ml of MEM containing 10% of the normal amount of monosodium phosphate.

Twenty-six ml of complete MEM (with low phosphate), containing 0.4% BSA instead of serum and 0.57 mc (22 μc/ml) carrier-free ^{32}P-phosphoric acid (International Chemical and Nuclear Corp.) was pipetted into each culture bottle. Due to the length of the labeling period, at 24 to 40 hours post infection (PI) another 0.57 mc of ^{32}P-phosphoric acid in 1.0 ml of complete MEM (with low phosphate and 0.4% BSA) was added to the medium. At 90 to 96 hours PI, the growth medium was collected and pooled.

Measurement of radioactivity. Two techniques commonly known as "spotting" and "plating" were used to prepare most samples for radioactivity measurements. A sample was "spotted" by absorbing a 10 μl aliquot into a dry membrane filter (Type B-6, 27 mm, Schleicher and Schuell Co.). The filter was dried in an oven at 60°C for 30 min., placed in a scintillation counting vial, and

immersed in 15 ml of scintillation fluor composed of 4% W/V 2,5-bis-[2-(5-tert-Butylbenzoxazolyl)]-Thiophene (BBOT, Packard Instrument Company, Inc.) in reagent grade toluene (Mallinckrodt Chemical Works).

"Plating" was performed using the same types of membrane filters, counting vials, and scintillation fluor used in "spotting." The plating procedure involved the following steps: (1) A sample, or aliquot from a sample, containing virus or nucleic acid was diluted to 1.0 ml with 0.01M Tris buffer, pH 7.4, containing 0.001M $MgCl_2$ (TML-buffer) in a Kahn tube, (2) the tube was placed in an ice bath and 0.1 ml of 100% trichloroacetic acid (TCA, Mallinckrodt Chemical Works) was added to give a final concentration of approximately 10% TCA, (3) after 10 to 15 min., the contents of the tube and five five-ml tube-rinses with cold 10% TCA were collected on a membrane filter under a vacuum, (4) the filter was rinsed five times with five-ml volumes of cold 10% TCA, and (5) the filter was dried, placed in a counting vial, and immersed in 15 ml of fluor.

A third technique was used for determining the radioactivity in 5-ml fractions obtained in anion exchange chromatography, and will be described below (see Anion exchange chromatography).

In all three cases, radioactivity measurements were made with a Packard Tri-Carb Liquid Scintillation Spectrometer,

Series 3000 (Packard Instrument Company, Inc.). Two channels with windows A to B and C to D were used. The base line discriminator controls A and C were set at 50 divisions, and the upper discriminator controls B and D were set at 1,000 divisions. Radioactivity measures for ^{32}P and ^{14}C were made using gains of 2.5% and 7.85%, respectively. Background radioactivity was determined by counting a vial with each set of samples that contained fluor only.

The counting periods used in radioactivity measurements were from one to ten minutes. Periods of one minute were used for counting samples from ^{32}P-labeled virus purification steps containing 3,000 to 400,000 counts per minute. Because determinations of radioactivity in samples from sucrose, cesium sulfate, or glycerol gradients were performed to locate labeled virus or nucleic acid, 10-minute counting periods were used for all samples regardless of their cpm. In addition, 10-minute counts were made for samples of fractions eluted from anion exchange columns containing 5 to 300 cpm (corrected for background), due to the large number of fractions (75 to 80 per experiment) and the short half-life of ^{32}P.

Radioactivity measurements of samples containing ^{32}P alone were made with both channels of the scintillation spectrometer; however, only the total counts from the A-B channel were used.

Each value was corrected by subtracting background radioactivity. Simultaneous determinations of ^{14}C and ^{32}P were performed using both channels at appropriate gain settings.

Virus purification. The same general procedures used to purify unlabeled virus were used for purification of ^{32}P-labeled virus. Growth medium from infected cell cultures was differentially centrifuged at 8, 700 x g for 10 min. (low speed) and 55, 000 x g for 60 min. (high speed). After nuclease treatment, the virus suspension in a volume of 1.8 ml was layered on a 10 to 55% sucrose gradient and was centrifuged at 42, 000 x g for two hours. One-ml fractions were collected through the bottom of the centrifuge tube. Aliquots of 0.1 ml and 20 µl were removed from each fraction for infectivity and radioactivity measurements, respectively. A drop from every fifth fraction was used for measuring the refractive index (see Determination of Virus Density).

Infectivity was determined using the end-point dilution method. Radioactivity was measured by "plating" the 20 µl aliquots and obtaining ^{32}P-counts per minute (cpm). ^{32}P-cpm and refractive indexes were graphically plotted versus fraction number, and the fractions in the peak of ^{32}P-radioactivity corresponding to the proper sucrose density (approximately 1.14 g/cm^3) were pooled (see Virus Purification, Rate-Zonal-Sucrose Gradient Centrification).

Phenol extraction. Nucleic acid was extracted from virus in the 6 to 8 ml of pooled sucrose-gradient fractions by the method of Robinson, Pitkanen, and Rubin (1965) as modified by Deeney (1969). The virus suspension was diluted approximately one to three with 0.01M Tris buffer, pH 8.8, containing 0.148M NaCl (TS-buffer) to reduce the sucrose concentration to about 10%. Sufficient 20% sodium dodecyl sulfate (SDS, recrystallized three times, Reagent grade, Sigma Chemicals) was added to give a concentration of 0.5% SDS. The suspension was stirred with a glass rod at room temperature for 10 min. to insure disruption of the virus particles, and was pipetted into a sterile Erlenmeyer flask in an ice bath. The cooled mixture was made to 10% dimethylsulfoxide (DMSO) by adding 100% DMSO (Grade one, Sigma Chemicals).

An equal volume of buffer-saturated phenol was combined with the SDS-treated virus mixture, and they were mixed on a reciprocating shaker for 15 min. at 4°C. The phenol used in this procedure was redistilled liquid phenol (Mallinckrodt Chemical Works) stored at -20°C, and thawed shortly before use. A buffer composed of 0.01M Tris buffer, pH 10.5, containing 0.148M NaCl was used to saturate the phenol.

The phenol-extracted mixture was dispensed into sterile 30-ml glass centrifuge tubes and centrifuged at 23, 300 x g for 10 min. Aqueous phases were removed with a Pasteur pipet and

placed in either a chilled flask for another phenol extraction, or a chilled separatory funnel for ether extraction. Remaining phenol phases were each washed once with five ml of TS-buffer, recentrifuged, and the aqueous phases obtained as before. A second phenol extraction was accomplished as described above, except the amount of saturated phenol used was one half the volume of the pooled aqueous phases from the first extraction.

Phenol was removed from the pooled aqueous phases by extracting four times with equal volumes of ether (anhydrous, J. T. Baker Chemical Co.). The nucleic acid in the final aqueous phase was precipitated by addition of two volumes of 95% ethanol and 0.1 volume of 20% sodium acetate, pH 5.0, and storage at -20°C for four to five hours. All of the precipitate was sedimented in one sterile, 30-ml glass centrifuge tube by centrifuging 25-ml volumes at 31,700 x g for 20 min.

The resulting pellet containing precipitated nucleic acid was dissolved in approximately 1.50 ml of TML or 2.0 ml TS-buffer at room temperature. An aliquot was removed, diluted to 1.0 ml with TML-buffer, and its absorption spectrum in the 230 to 290 mμ UV region was determined as described for unlabeled viral nucleic acid. This sample was then "plated" and ^{32}P-radioactivity measured. The remaining nucleic acid solution was dispensed into Kahn tubes in small volumes, stoppered, and frozen at -70°C; or, it was

subjected to cesium sulfate gradient centrifugation.

Cesium sulfate gradient centrifugation. Equilibrium centrifugation of extracted ^{32}P-labeled viral nucleic acid in cesium sulfate was performed according to the technique described by Robinson et al. (1964). A solution containing approximately 5 μg of nucleic acid was diluted to 1.52 ml with TML-buffer and mixed with 1.98 ml of saturated cesium sulfate in a 1/2-by-2 inch nitrocellulose centrifuge tube (Beckman Instruments, Inc.). The solution had a density of approximately 1.600 g/cm^3. Two ml of mineral oil was layered on the cesium sulfate solution and the gradient was formed by centrifugation in an SW-50 or SW-39 head at 130,000 x g for 60 hours at 22°C. Fractions of 0.17 and 0.19 ml were collected from the bottom of the tube. Every fifth fraction was 0.19 ml, and one drop from each of these was used for refractive index determination. Each fraction was diluted to one ml with TML-buffer, absorbance was measured at 260 and 280 mμ, and radioactivity was determined by liquid scintillation of "plated" samples.

Saturated cesium sulfate (99.7% Penn Rare Metals, Inc.) was prepared in double distilled water and membrane (Type B-6, 27 mm, Schleicher and Schuell Co.) filtered twice while hot. A standard curve of density versus refractive index was determined using TML-buffer as diluent.

Glycerol gradient centrifugation. Extracted viral nucleic acid was sedimented in a glycerol gradient in order to determine the sedimentation coefficients and homogeneity of the nucleic acid molecules. Five-ml, 10 to 30% glycerol gradients in 0.01M Tris buffer, pH 7.4, containing 0.1M NaCl and 0.001M EDTA (TSE-buffer) were prepared in a 1/2-by-2 inch nitrocellulose tubes with a Buchler gradient marker. A solution containing ^{32}P-labeled OSV nucleic acid and ^{14}C-labeled myeloblast cell RNA, with approximately 20,000 cpm each, in 0.5 ml of TSE-buffer was layered on a gradient. Centrifugation was performed in an SW-65 rotor at 179,000 x g for 2.5 hours at 3°C. Fractions of 0.2 ml were collected through the bottom of the centrifuge tube, diluted to 1.0 ml with TSE-buffer, and the bottom of the empty tube was rinsed with 1.0 ml of TSE-buffer.

A 0.5 ml aliquot from each fraction and the tube rinse were "plated," and ^{14}C- and ^{32}P-radioactivities were measured simultaneously using two channels of the liquid scintillation counter. Counts per minute of ^{14}C and ^{32}P were plotted versus fraction number. Sedimentation coefficients were estimated by using the 28S and 18S ribosomal RNA peaks of ^{14}C-myeloblast cell RNA (Watson, 1970) as references. The remaining 0.5 ml of each fraction was mixed with 0.1 ml of a 100 μg/ml RNase solution in 0.01M Tris buffer and incubated at 30°C for 40 min.

Nuclease-treated samples were "plated" and radioactivity measured in the same manner as the untreated samples.

Anion exchange chromatography. Nucleotide composition of the ^{32}P-labeled viral nucleic acid banded in cesium sulfate gradients was determined with anion exchange chromatography using the method of Hayashi and Spiegelman (1961). The fraction containing the greatest ^{32}P-radioactivity was divided into three equal volumes of about 0.3 ml. Each volume was combined with 1.5 mg of unlabeled bulk Escherichia coli RNA in 0.072 ml of TS-buffer (kindly supplied by Dr. George S. Beaudreau, Department of Agricultural Chemistry, Oregon State University). The three RNA solutions were each diluted to 1.0 ml, and the RNA was precipitated by adding 0.1 ml of 100% TCA to each. After collecting the precipitates on membrane filters, the membranes were rinsed three times with 1-ml volumes of 0.3N potassium hydroxide (KOH), and the pooled rinses were incubated at 37°C for 18 hours.

Chromatography was performed with Dowex 1-x-8 (100-200 mesh, chloride form, Bio-Rad Laboratories) converted to the formate form. A three-ml sample of hydrolyzed RNA in 0.3N KOH was adjusted to pH 9.5 with 88% formic acid (Reagent grade, Mallinckrodt Chemical Works), and loaded on a 0.87 x 5.0 cm column. The eluting solvents, the approximate volumes, and the order in which they were used were as follows: double-distilled

water (25 ml); 0.005N formic acid (50 ml); 0.025 N formic acid (50 ml); 0.01N formic acid (100 ml); 0.05N formic acid and 0.05N ammonium formate (Reagent grade, J. T. Baker Chemical Co.) (75 ml); and 0.1N formic acid and 0.2N ammonium formate (75 ml).

Five-ml fractions were collected with an automatic fraction collector (Fracto-Mette 200, Buchler Instruments, Inc.). The absorbance of each fraction was measured at 260 mμ. ^{32}P-radioactivity was determined by evaporating the five-ml fractions to dryness in glass counting vials at 225°F, adding 15 ml of scintillation fluor to each vial, and measuring radioactivity in a liquid scintillation spectrometer. (The method of calculating nucleotide composition will be described in the Results section.)

Immunological Techniques

The antigenic relationships between OSV, SRCD virus, and IHN virus were investigated with cross-plaque-neutralization tests. Rabbit antisera against the three viruses were tested with each virus, and the serum titers were expressed as 50% neutralization end points.

Preparation of antisera

Rabbits were immunized with a concentrated, purified preparation of one of the three viruses propagated in the cell line

SSE-30. (Two rabbits were used for each virus.) Propagation and purification of the three viruses were performed in the same manner as described for ^{32}P-labeled virus used for nucleic acid extractions (see Characterization of Viral Nucleic Acid, ^{32}P-labeled Virus), with the following exceptions: (1) nuclease treatment and rate-zonal sucrose gradient centrifugation were omitted, (2) differential centrifugation involved a low-speed cycle of 2, 200 x g for 30 min, and (3) virus pellets were resuspended in MEM to give between 5×10^8 and 2×10^9 $TCID_{50}$/ml.

For the first series of injections, rabbits received one one-ml intravenous injection of undiluted virus suspension in MEM and two one-ml intramuscular injections of a sterile emulsion consisting of equal volumes of virus suspension and Complete Freund's adjuvant (Difco). The emulsion was prepared using two "ganged" 5 cc Luer-Lok-type syringes (Becton, Dickinson and Co.) (Williams and Chase, 1967). Two 15-guage stainless steel needles, 1.5 inches in length, joined by a 2-inch piece of Tygon tubing, were used to connect the syringes. One syringe received one to three ml of adjuvant, and the other syringe received an equal volume of virus suspension. After expelling the air from each syringe, they were connected with the emulsion formed by forcing the syringe plungers back and forth 30 to 40 times. An emulsion was tested for stability by storing the

"ganged" syringes at 4°C for 15 to 30 min.; if the emulsion had not separated by this time, it was considered formed. (In preliminary trials, the method described above produced emulsions which remained intact when drops were placed on water. The emulsion also contained evenly dispersed, stable water droplets when examined with a microscope.)

A second series of rabbit injections was performed approximately 14 days after the first series. Each rabbit received two 1.0-ml intramuscular injections of a sterile emulsion containing one-to-one proportions of virus suspension and Complete Freund's adjuvant prepared as described above.

Two weeks after the second rabbit injection series, trial bleedings were performed. Blood was collected from the ear into a sterile 12-ml conical centrifuge tube. A clot was allowed to form at room temperature for one hour and was freed from the sides of the tube with a wooden application stick. The tube was stored overnight at 4°C in order to constrict the clot. The serum was pipetted from this tube into another sterile, 12-ml, conical centrifuge tube and centrifuged at 300 x g for 15 min. Sterilization of the clarified rabbit serum was performed by Millipore filtration (average pore size, 0.22μ) with vacuum. Sterile serum was stored at 4°C for up to two months and periodically checked for sterility by inoculation into Thioglycollate broth. Shortly before each plaque neutralization test,

a sample of serum was heated at 56°C for 30 min. Normal rabbit serum was treated in the same manner.

Rabbit immune serum obtained from a trial bleeding was tested for virus-neutralizing activity against the homologous virus, using the plaque neutralization test described below. Immune serum used for cross-plaque neutralization tests against all three viruses was obtained approximately three weeks after the second series of rabbit injections, and treated in the same manner as serum obtained from trial bleedings.

Cross-plaque-neutralization test

Each cross-plaque-neutralization test was essentially three plaque neutralization tests of one antiserum against each of the three viruses. At the beginning of each test, the antiserum was diluted with MEM containing 0.4% BSA, pH 7.4 (MEM-diluent) to give five two-fold serum dilutions which had previously been shown to cause between 10 and 90% plaque neutralization with the homologous virus. Each of the five dilutions of antiserum was dispensed into three sets of sterile, screw-cap 17 x 150 mm tubes, 1 ml per tube. The screw-caps were replaced with sterile latex stoppers (West Co.), and the tubes were stored at 4°C for 30 min. to 8 hours until needed.

Included with each set of antiserum dilutions was a tube containing 1.0 ml of normal rabbit serum dilution in MEM-diluent. The dilution of the normal serum was the same as the lowest dilution of

the antiserum being tested; however, normal serum was not diluted any greater than 1/200. (I.e., if the lowest dilution of antiserum was 1/1000, then the dilution of normal serum was 1/200.)

After the serum dilutions were prepared, dispensed, and stored at 4°C, a tube of stock virus frozen at -60°C (see Viruses) was thawed and diluted in MEM-diluent to give approximately 100 pfu/0.15 ml. The proper dilution giving the desired number of plaques under the conditions of the plaque neutralization test had been previously determined. One ml of this virus dilution was pipetted into each of the six tubes in one set of serum dilutions. The tubes were mixed and incubated at 18°C for 60 min. Unneutralized virus was assayed for exactly as described for the monolayer-plaque-assay technique, using 2-oz prescription bottles containing confluent monolayers of cell line CHSE-214 (see Infectivity Assays). Each of three replicate bottles received 0.3 ml of the same serum-virus mixture. All mixtures were similarly assayed in triplicate. At two-hour intervals, this process was repeated with the other two viruses using the sets of serum dilutions stored at 4°C.

The antiserum dilution giving 50% plaque neutralization was determined for each virus. Percent plaque neutralization for each serum dilution was calculated using the equation

$$100 - 100 \times \frac{\text{average pfu/bottle for a single antiserum dilution}}{\text{average pfu/bottle for the control with normal serum}}.$$

The percent plaque neutralization values between 10 and 90% were plotted versus the corresponding dilutions on semi-logarithmic graph paper (percentages on the $\log_{10}$ scale and dilutions on the linear scale). A line was drawn which best fit the set of three or more points. The 50% neutralization end point was the antiserum dilution at the point on the line corresponding to 50% plaque neutralization.

Electron Microscopy

An electron microscope was used to examine negatively stained suspensions of OSV. Virus was propagated and differentially centrifuged in a manner similar to that described for viral antigen production, except low-speed centrifugation was at 19,600 x g for 15 min. Virus pellets were suspended in either five percent sucrose in 0.005M EDTA, or one percent ammonium acetate. Virus suspensions containing approximately 2×10^9 pfu/ml were placed on Formvar-coated copper grids (200 mesh, Ladd Research Industries) by one of two methods.

One procedure involved mixing virus suspended in sucrose-EDTA solution with an equal volume of one percent glutaraldehyde (K and K Laboratories). This suspension was allowed to stand at $4^{\circ}C$ for 20 min. A drop of fixed virus was placed on a grid for five min. and the excess fluid was removed with filter paper. Without allowing

the suspension to dry, the virus was stained for about five seconds with a drop of one percent phosphotungstic acid (PTA), pH 6.5, and the drop was removed with filter paper. In the other method, virus in one percent ammonium acetate was mixed one to one with one or two percent PTA, pH 6.5, and was sprayed onto carbon-stabilized Formvar-coated grids with a glass nebulizer. Both fixed and unfixed virus preparations were examined immediately with a Philips EM300 electron microscope at 60kV with direct magnifications of 14,000 and 53,000.

EXPERIMENTAL RESULTS

Virus Purification

Each of the experimental procedures used to characterize the OSV required concentrated, purified virus suspensions. Virus purification techniques were developed to satisfy the requirements of each procedure.

Infectious cell culture medium was subjected to one cycle of differential centrifugation in order to obtain virus suspensions for virus density determination, electron microscope examination, and rabbit immunization. Table 1 compares three variations of the differential centrifugation cycle. Each method differed from the other two by the conditions used for low-speed centrifugation (Step 1), and the solutions used to resuspend the virus pellets after centrifugation at 55, 000 x g for 60 min (Step 2; see Table 1).

Method 1 yielded a concentrated virus suspension (Step 2) with 60% of the virus infectivity initially found in the culture medium. However, 79% of this infectivity was sedimented at 130 x g for 10 min (Step 3) and was completely recovered in the resulting pellet. These results indicated that during the high-speed centrifugation cycle, 80 to 90% of the virus was aggregated or complexed with cellular debris, making it more easily sedimented.

Table 1. A comparison of three differential centrifugation techniques for virus purification.

Method Number	Time and Centrifugal Force Used in Low-Speed Cycle: Time (min)	Time and Centrifugal Force Used in Low-Speed Cycle: Centrifugal Force x g	Infectivity of Samples: Step 1: Pooled Cell Culture Medium Total $TCID_{50}$	Step 1: Supernate After Low-Speed Centrifugation Total $TCID_{50}$	Step 1: Supernate After Low-Speed Centrifugation % Recovery[a]	Step 2: Suspended Virus[b] Pellets After High-Speed Centrifugation Total $TCID_{50}$	Step 2: Suspended Virus[b] Pellets After High-Speed Centrifugation % Recovery	Step 3: Supernate After Centrifugation of Concentrated Virus at 130 x g for 10 min. Total $TCID_{50}$	Step 3: Supernate After Centrifugation of Concentrated Virus at 130 x g for 10 min. % Recovery	Overall Percent Recovery of $TCID_{50}$ for each Method
1	30	2,200	3.2×10^9	2.3×10^9	72	1.9×10^9	83	4.0×10^8	21	13
2	15	8,700	1.8×10^8	5.6×10^7	31	4.2×10^7	75	4.0×10^7	100	12
3	15	19,600	2.9×10^9	5.4×10^8	21	1.6×10^8	30	1.6×10^8	100	6

[a] Percent recovery of $TCID_{50}$ from the preceding step

[b] Virus pellets in Step 2 were suspended in three different solutions, TBE-buffer (Method 1); 0.05M Tris buffer, pH 7.0, with 0.001M $MgCl_2$ and 30 ug/ml each RNase and DNase (Method 2); and 5.0% sucrose with 0.005M EDTA (Method 3).

Table 1 shows the results of two other differential centrifugation methods which were used in an effort to remove more host-cell material from the cell culture fluid prior to the high-speed cycle. Although both methods yielded concentrated virus suspensions unaffected by centrifugation at 130 x g for 10 min, the overall recovery of virus infectivity was as low as or lower than the recovery obtained by the method described above.

Disodium EDTA was added to infectious cell culture fluid in an effort to dissociate virus-cell debris complexes. Sufficient 8.4% disodium EDTA in double distilled water, adjusted to pH 7.4 with solid Tris, was added to pooled culture fluid to give a final concentration of 0.75%. No improvement in the recovery of virus infectivity was observed when the EDTA-treated culture medium was differentially centrifuged at 8, 700 x g for 15 min (low speed) and 55, 000 x g for 60 min (high speed).

The results of increasing the centrifugal force of the low-speed cycle and adding EDTA to the culture fluid indicated that other means were required to reduce the amount of virus-cell debris complexes formed during differential centrifugation. With the assumption that pelleting the virus in the presence of cell debris on the sides of polycarbonate centrifuge tubes caused a large amount of complex formation, a modified differential centrifugation technique employing 95% glycerol pads was tried. Table 2 contains the

Table 2. Characteristics of a virus purification scheme utilizing 95% glycerol pads during initial virus concentration (see METHODS AND MATERIALS, Virus Purification, Differential centrifugation).

Purification Step Number	Sample	Vol. (ml)	$TCID_{50}$ per ml	Mg protein per ml	$TCID_{50}$ per mg of protein	Percent recovery stepwise of $TCID_{50}$
1	Pooled infective culture medium	300.0	1.2×10^7	4.80	2.5×10^6	---
2	Supernate from low-speed centrifugation at 2,200 x g for 30 min	300.0	1.2×10^7	4.30	2.8×10^6	100
3	Pooled and diluted layers from three 95% glycerol pads after initial virus sedimentation at 42,000 x g for 60 min.	25.0	2.1×10^8	1.06	2.0×10^8	175
4	Diluted layer from one 95% glycerol pad containing virus sedimented in the presence of 40% glycerol at 42,000 x g for 120 min.	25.0	2.1×10^8	0.30	7.0×10^8	100
5	Supernate after centrifuging the suspension in Step 4 at 2,200 x g for 30 min.	25.0	7.5×10^7	0.18	8.3×10^7	36
6	Suspended pellet after high-speed centrifugation at 55,000 x g for 60 min, and clarification at 130 x g for 10 min.	1.0	2.4×10^9	ND*	--------	128
7	Virus band after centrifugation in a 10 to 60% sucrose gradient at 42,000 x g for two hours.	5.0	7.5×10^7	0.02	3.75×10^9	16

*Not done

results of one experiment using this method. Steps one through three were essentially the same as the first differential centrifugation procedure described in Table 1, except the virus was sedimented onto 95% glycerol pads. The concentrated virus from step three was centrifuged in the presence of 40% glycerol onto another 6-ml 95% glycerol pad in order to eliminate as much cell debris and protein as possible from the virus suspension. The resulting virus suspension was diluted to 25 ml with TBE-buffer and centrifuged at 2,200 x g for 30 min to remove virus-cell debris complexes (steps four and five). The virus in the supernate from step five was pelleted, suspended in 1.0 ml of TBE-buffer, and this suspension was centrifuged at 130 x g for 10 min (step six). Sixty-eight percent of the virus initially found in the culture medium was recovered in the suspension from step six.

Although the results of the procedure described in Table 2 demonstrated the importance of eliminating cell debris before pelleting the OSV during differential centrifugation, the method was too laborious and unpredictable to warrant its use for routine experimental procedures. For example, a tube slicer was required to quantitatively remove sedimented virus from glycerol pads in 1-x-3-inch nitrocellulose tubes; and its use on this size tube caused severe tube distortion and in several cases virus layers were lost due to leakage around the tube.

Differential centrifugation was the first of three purification

procedures used to purify OSV for nucleic acid extraction. The other two procedures were nuclease treatment and rate-zonal sucrose gradient centrifugation. Nuclease treatment was performed by suspending virus pellets resulting from differential centrifugation in a solution of RNase and DNase containing 30 μg of each per ml, and incubating the suspension at 25°C for one hour. No significant loss of virus infectivity was observed during nuclease treatment.

The effect of rate-zonal sucrose gradient centrifugation on recovery of virus infectivity is displayed in Tables 2 and 3. The virus suspension resulting from differential centrifugation in Table 2, containing 68% of the initial virus infectivity, was layered on a 10 to 60% sucrose gradient. After centrifuging the gradient at 42,000 x g for two hours, 2.0-ml fractions were collected, and the fractions containing the virus band were pooled (step 7). Sixteen percent of the virus infectivity in the suspension layered on the gradient was recovered in the virus band. The virus band contained 10% of the infectivity initially detected in the culture medium; however, in terms of $TCID_{50}$/mg of protein, the virus suspension from the gradient was 1,500 times more pure than the virus in the original culture medium.

Table 3 contains the results of a virus purification procedure used to purify ^{32}P-labeled virus for nucleic acid extraction. Differential centrifugation consisted of a low-speed cycle of 8,700 x

Table 3. Characteristics of the virus purification procedure used to purify unlabeled and ^{32}P-labeled virus for nucleic acid extraction.

Sample	Vol. (ml)	$TCID_{50}$ per ml	mg protein per ml	^{32}P-cpm per ml	$TCID_{50}$ per mg of protein	Percent Stepwise Recovery from the Value Determined in the Previous Step $TCID_{50}$	Percent Stepwise Recovery from the Value Determined in the Previous Step ^{32}P-cpm
Pooled Culture Medium	125.0	1.6×10^8	4.5	ND	3.6×10^7	---	---
Supernate from Low-Speed Centrifugation	125.0	1.2×10^8	4.7	2.86×10^5	1.7×10^7	75	---
Resuspended Pellet* After High-Speed Centrifugation and Nuclease Treatment	1.8	1.2×10^{10}	ND	8.32×10^6	ND	144	42
Pooled Fractions from the Sucrose Gradient Containing the Virus Band	6.0	1.0×10^9	0.55	1.09×10^6	1.9×10^9	28	44

*Pellet from high-speed centrifugation was suspended in 0.9 ml each of 60 μg/ml RNase and 60 μg/ml DNase.

g for 15 min and a high-speed cycle of 55, 000 x g for 60 min. There was no loss of virus infectivity during these centrifugation cycles and the nuclease treatment. Approximately 30% of virus infectivity in the suspension layered on the sucrose gradient was detected in the pooled gradient fractions containing the virus band. The cumulative recovery of virus infectivity in this purification procedure was 30%, and the $TCID_{50}$/mg of protein ratio of the final virus suspension was 53 times that of the infective culture medium. Although the purification scheme in Table 3 resulted in a smaller increase in virus purity than the scheme in Table 2, the ratios of $TCID_{50}$/mg of protein in the final virus suspensions were similar, i.e., 1.9×10^9 (Table 3) and 3.75×10^9 (Table 2).

In addition to the 70 to 80% loss of virus infectivity resulting from banding OSV in a sucrose gradient, a similar loss occurred while removing sucrose from the virus band. Pooled gradient fractions with the virus band contained approximately 38% sucrose. Sucrose removal was necessary if the nucleic acid extracted from banded virus was to be used in colorimetric tests for ribose and deoxyribose. Also, for electron microscope observations of banded virus, the sucrose concentration was reduced below 5%.

Parts of three representative experiments demonstrating methods used for sucrose removal are described in Table 4. The sucrose concentration of the final virus suspension in Experiment A

Table 4. Descriptions and results of three methods used for removing sucrose from pooled sucrose gradient fractions containing banded virus.

Experiment	Sample	Vol. (ml)	Total $TCID_{50}$	Percent infectivity recovered
A	Pooled sucrose gradient fractions	5.0	4.0×10^8	---
	Suspended virus pellet* after diluting pooled fractions to 25 ml with TBE-buffer and centrifugation at 55,000 x g for 60 min.	0.6	1.3×10^8	32
B	Pooled sucrose gradient fractions	4.0	2.3×10^8	---
	Suspended virus pellet* after diluting pooled fractions to 50 ml with TBE-buffer, centrifugation at 55,000 x g for 60 min., resuspension in 25 ml of TBE-buffer, dialysis against TBE-buffer for 25 hrs., and recentrifugation at 55,000 x g for 60 min.	2.0	3.2×10^7	15
C	Pooled sucrose gradient fractions	5.0	3.2×10^8	---
	Suspended virus pellet* after diluting pooled fractions to 25 ml with 25% sucrose, dialysis against gradually decreasing concentrations of sucrose for 34 hrs., and centrifugation at 55,000 x g for 60 min.	0.4	$<3.2 \times 10^7$	<10

* Virus pellets were suspended in TBE-buffer.

was approximately 2.5%, because this suspension was used for electron microscope observations. Similar suspensions in Experiments B and C were used for nucleic acid extraction and contained less than 5 μg of sucrose per ml.

The principal differences between Experiments B and C were the solutions used to dilute and dialyze the pooled gradient fractions. In Experiment B, dilutions were made with TBE-buffer. Twenty-five ml of a diluted virus suspension was dialyzed against one liter of TBE-buffer at 4°C with buffer changes at 8 and 21 hours. Only one dilution of the pooled fractions was performed in Experiment C using 25% sucrose in TBE-buffer. This 25-ml suspension was dialyzed at 4°C against one-liter volumes of gradually decreasing concentrations of sucrose in TBE-buffer as follows: 15% for 12 hours, 10% for 5 hours, 5% for 10 hours, and TBE-buffer alone for 5 hours.

Due to the combined loss of virus infectivity during rate-zonal sucrose gradient centrifugation and the sucrose removal techniques described in Table 4 (Experiments B and C), the overall recovery of infectivity was extremely low. For example, the final virus suspension resulting from the purification procedure outlined in Table 3 and Table 4 (Experiment B) contained approximately 0.2% of the virus infectivity found in the original infectious culture medium.

The sucrose removal step was eliminated from the purification procedure presented in Table 3 when ^{32}P-labeled OSV was purified. Since colorimetric tests were not performed on the ^{32}P-labeled nucleic acid extracted from the pooled sucrose gradient fractions, the removal of sucrose was not necessary.

Determination of OSV Density

The results of three separate density determination experiments are shown in Figure 1. OSV used in these experiments was propagated and purified as described in METHODS AND MATERIALS (see Determination of Virus Density). Two ml of concentrated and purified virus suspended in 0.05M Tris buffer, pH 7.4, was layered onto a 10 to 60% (W/V) sucrose gradient. Gradients were prepared by layering a linear 10 to 55% sucrose gradient onto a 2.0 ml 60% sucrose pad with a Buchler gradient maker. Sucrose solutions used for density determinations were dissolved in double distilled water.

In Figures 1a, 1b and 1c, the mid-point of the predominant peak of virus infectivity was located at a sucrose density of 1.16 g/cm^3. The gradient in Figure 1a contained two additional infectivity peaks. The lower peak was in the region of the gradient with a density of 1.20 g/cm^3, and the sucrose density corresponding to the location of the upper peak was 1.10 g/cm^3. Because the volume of the fractions collected from the gradient represented in Figure 1b was

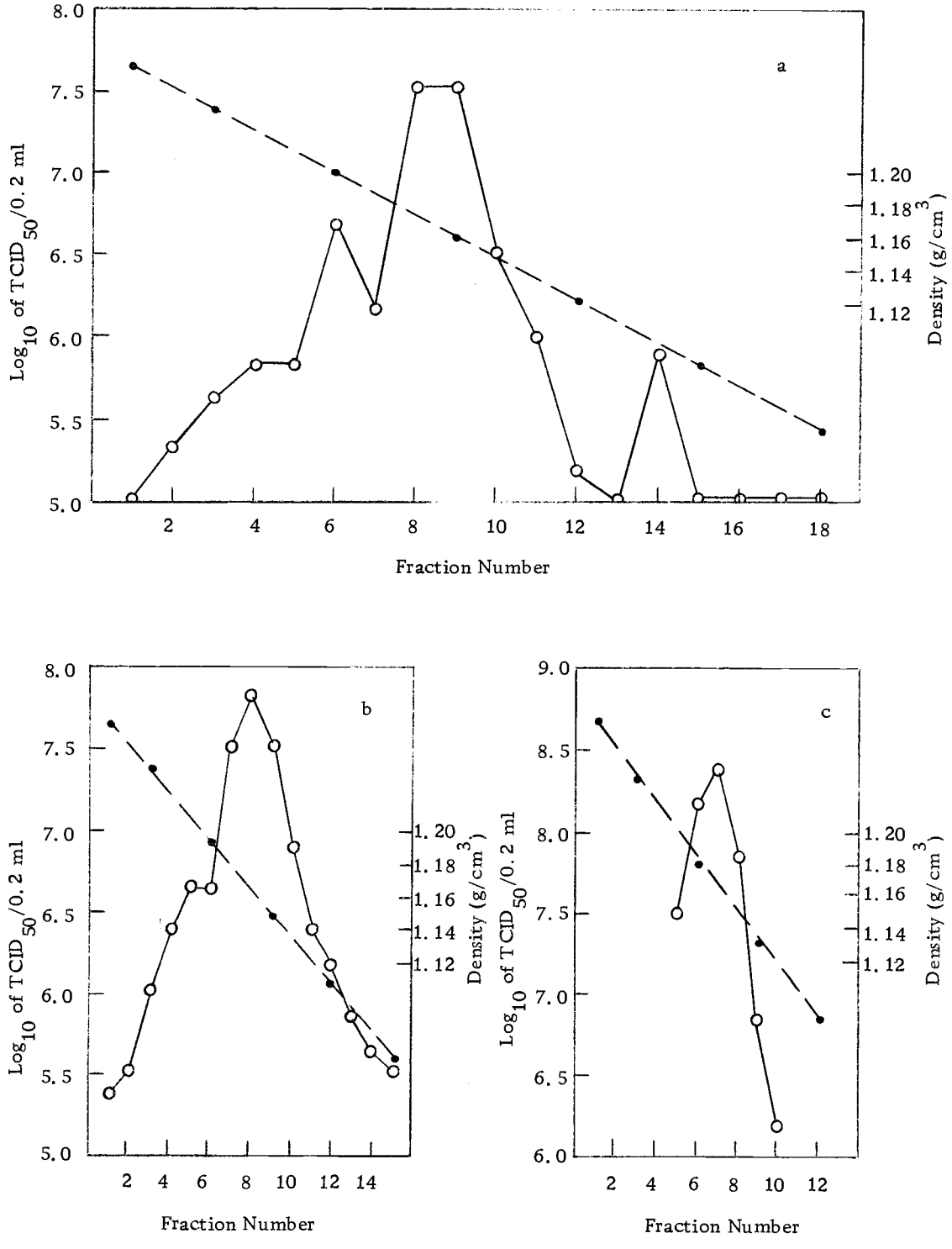


Figure 1. Equilibrium centrifugation in a sucrose gradient of OSV. Ten to 60% (w/v) linear gradients were centrifuged at 42,000 x g for 16 hours. Figures 1a, 1b, and 1c represent three different experiments. Fractions in 1a were 1.7 ml, and fractions in 1b and 1c were 2.0 ml. o, $\log_{10}$ of $TCID_{50}$; •, density

2.0 ml (120 drops) instead of the 1.7 ml (110 drops) volume of the fractions in Figure 1a, the two additional infectivity peaks are less apparent. However, shoulders of infectivity are present above and below the predominant infectivity peak in Figure 1b, and they are at the same sucrose densities observed for the upper and lower peaks in Figure 1a. The results of the virus density determination shown in Figure 1c were intended to confirm previously determined sucrose density values found for the major infectivity peak; thus, only the fractions comprising this peak were assayed for infectivity.

Each of the peaks of virus infectivity described above corresponded to the positions of visible bands in the sucrose gradients. The lower band was usually 10 mm wide and contained clumps of material as large as 1 mm in diameter. This band became stationary after centrifugation for approximately two hours. The middle peak containing most infectivity corresponded to two visible bands, each with a width of 2 mm, and approximately 3 mm apart. These two closely associated bands were homogenous and sedimented at the same rate, reaching equilibrium after approximately eight hours of centrifugation.

The photograph in Figure 4 demonstrates the appearance of the visible bands in a sucrose gradient. This gradient was prepared with 10 to 55% sucrose and was centrifuged for two hours at 42, 000 x g as part of the purification procedure used to purify ^{32}P-labeled

virus for nucleic acid extraction.

Characterization of OSV Nucleic Acid

Unlabeled virus

The concentration and type of nucleic acid in perchloric acid (PCA) extracts of purified suspensions of OSV were determined spectrophotometrically. Determinations were performed on nucleic acid solutions obtained from three separate experiments. The results of the virus purification procedures are given in Table 5-A. Infectious cell culture medium was first subjected to one cycle of differential centrifugation (low-speed, 2, 200 x g for 30 min; high-speed, 55, 000 x g for 60 min). Nuclease treatment and rate-zonal sucrose gradient centrifugation were the same as described for the procedure outlined in Table 3. The virus band was dialyzed against TBE-buffer to reduce the sucrose concentration to less than 5 μg/ml by the technique given in Table 4 (Experiment B). Pellets of dialyzed virus from each purification procedure were suspended in a final volume of 3. 0 ml of 0. 5N PCA.

Nucleic acid was extracted twice by incubating the initial 3. 0-ml suspension in 0. 5N PCA at 70°C for 60 min, removing the supernate after centrifugation at 500 x g for 20 min, and re-extracting the pelleted material in 1. 0 ml of 0. 5N PCA by the

Table 5. The results of three experiments involving purification of unlabeled OSV, extraction of viral nucleic acid, and spectrophotometric determinations of total nucleic acid, RNA, and DNA concentrations.

A. Virus purification data

Sample	Volume (ml) Experiment No. I	II	III	$TCID_{50}$ per ml Experiment No. I	II	III
Culture medium	100	120	180	1.6×10^8	3.2×10^8	5.0×10^8
Virus suspension after differential centrifugation and nuclease treatment	2.0	3.0	3.0	2.4×10^8	2.2×10^9	2.1×10^9
Virus band after sucrose gradient centrifugation	2.0	4.0	5.0	1.6×10^7*	3.8×10^7	1.0×10^8

*Infectivity in Experiment I is given for the virus band after dialysis.

B. Spectrophotometry data

Experiment No.	Concentration in µg/ml in PCA extracts of RNA (orcinol test)	DNA (diphenylamine test)	Total nucleic acid (From absorbance at 260mµ)	Absorbance ratio at 260/280 µm in PCA extracts
I	3.48	0	2.7	1.25
II	2.93	0.2	2.0	1.37
III	7.82	0.4	5.0	1.27

above method. The combined supernatants were tested for total nucleic acid content by measuring absorbance at 260 mμ (see Table 5-B). Also, the absorption spectrum from 230 to 290 mμ was obtained by measuring absorbance at 10 mμ increments. A representative spectrum is shown in Figure 2a, and the 260/280 mμ ratios of each experiment are given in Table 5-B.

After each PCA extract was used for UV determinations, it was divided into two equal volumes. The concentration of RNA in one volume was determined with the orcinol reaction, using simultaneous analyses of RNA and sucrose. Figures 2b and 2c demonstrate the necessity for simultaneous determinations. Figure 2b shows a typical absorption spectrum of an orcinol reaction mixture containing PCA-extracted OSV nucleic acid. The same absorption maxima at 550 and 675 mμ observed in this figure were observed in the absorption spectra of orcinol reaction mixtures containing sucrose and yeast RNA, respectively, presented in Figure 2c.

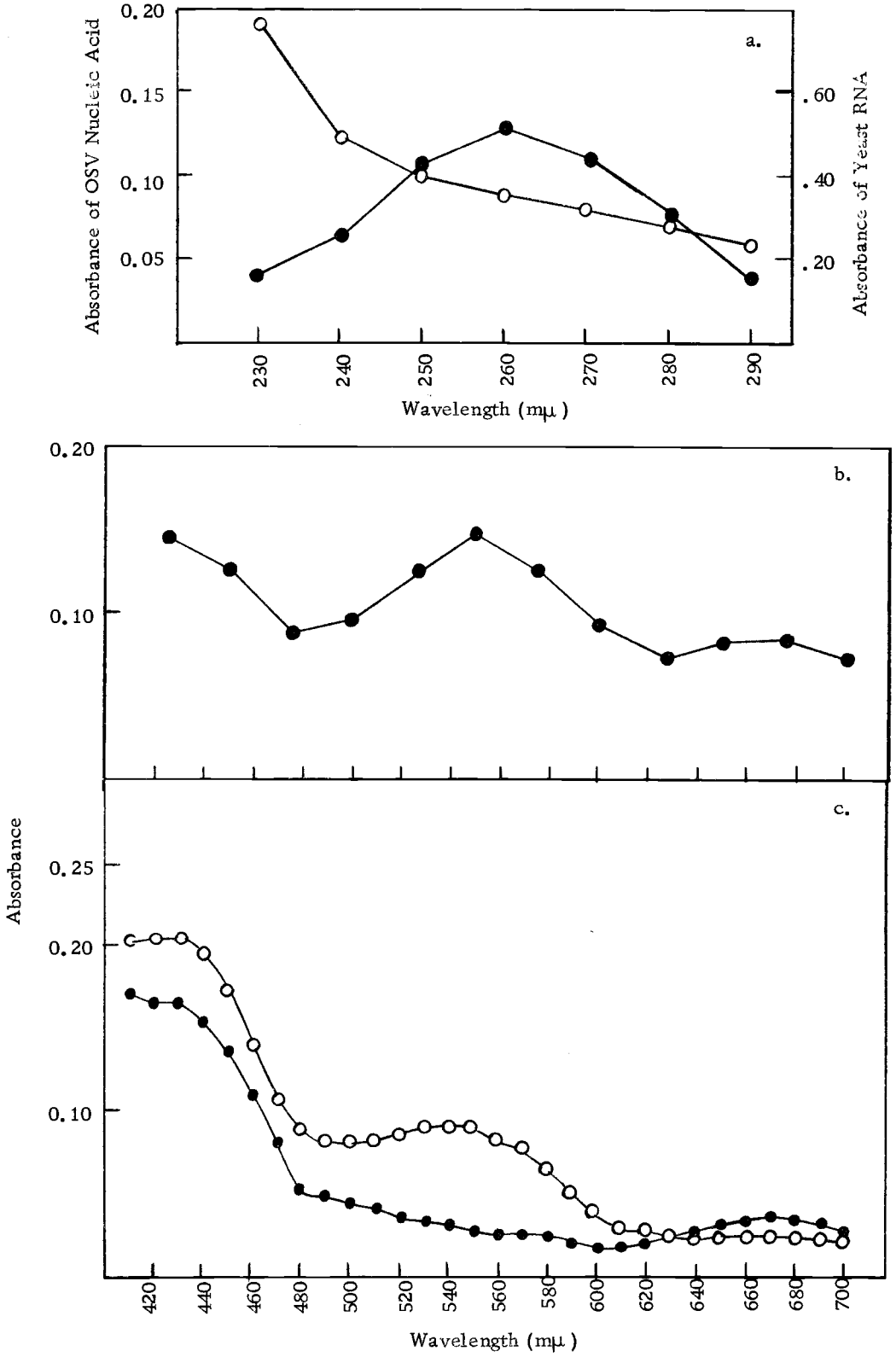
Known concentrations of sucrose and yeast RNA were tested with the orcinol reaction and standard curves at wavelengths of 550 and 675 mμ were prepared. The molar absorbtivity of each compound at each wavelength was obtained by determining the slopes of the standard curves. The molar absorbtivities for RNA at 550 and 675 mμ were 2.67×10^{-3} and 8.90×10^{-3}, respectively. For sucrose concentrations at 550 and 675 mμ, the molar absorbtivities

Figure 2. Spectrophotometric characteristics of PCA-extracted, unlabeled, OSV nucleic acid.

a. Absorption spectra of a 0.5N PCA solution containing PCA-extracted, unlabeled nucleic acid (o—o) and a 0.5N PCA solution with 20 μg/ml yeast RNA (●—●).

b. Absorption spectrum of an orcinol reaction mixture containing PCA-extracted, unlabeled OSV nucleic acid.

c. Absorption spectra of two orcinol reaction mixtures, one containing 2.5 μg/ml sucrose (o—o) and the other with 2.0 μg/ml yeast RNA (●—●).


a.
Absorbance of OSV Nucleic Acid
Absorbance of Yeast RNA
0.20
0.15
0.10
0.05
.60
.40
.20
230
240
250
260
270
280
290
Wavelength (mμ)
b.
0.20
0.10
c.
Absorbance
0.25
0.20
0.10
420
440
460
480
500
520
540
560
580
600
620
640
660
680
700
Wavelength (mμ)

were 2.50×10^{-2} and 2.86×10^{-3}, respectively.

Simultaneous analyses were performed by first determining the absorbances (A) at 550 and 675 mμ of an orcinol test mixture containing PCA-extracted OSV nucleic acid. These values were substituted in the following equations, which were then solved simultaneously:

$$\mu g/ml\ RNA = \frac{A_{675\ m\mu} - (2.86 \times 10^{-3})(\mu g/ml\ sucrose)}{8.90 \times 10^{-3}}$$

$$\mu g/ml\ sucrose = \frac{A_{550\ m\mu} - (2.67 \times 10^{-3})\ (\mu g/ml\ RNA)}{2.50 \times 10^{-2}}$$

For example, the concentration of RNA in the PCA extract (3.48 μg/ml) from Experiment I presented in Table 5-B was determined as follows:

(1) $A_{550\ m\mu} = 0.07 \qquad A_{675\ m\mu} = 0.023$

$$\mu g/ml\ suc. = \frac{(0.07)(0.0089) - (0.00267)(0.023) + (0.00286)(\mu g/ml\ suc.)(0.00267)}{(0.0089)(0.025)}$$

$$= \frac{5.63 \times 10^{-4}}{2.15 \times 10^{-4}} = 2.62\ \mu g/ml$$

$$\mu g/ml\ RNA = \frac{0.023 - (0.00286)(2.62\ \mu g/ml\ sucrose)}{0.0089}$$

$$= \frac{0.00155}{0.0089} = 1.74\ \mu g/ml$$

(2) Since the volume of PCA extract used in the orcinol test was one half the total volume, the final concentration of RNA per ml in the total extract was (2)(1.74 μg/ml) or 3.48 μg/ml.

The other portion of each PCA extract containing OSV nucleic acid was assayed for DNA content, using the diphenylamine reaction. Table 5-B presents the results of orcinol and diphenylamine tests performed on each of the three PCA-extracts. In all three cases, the RNA concentration was at least 14 times greater than the concentration of DNA. Also, RNA concentrations corresponded more with the total nucleic acid content of each PCA extract than did the concentration values of DNA. Therefore, the data in Table 5-B provide evidence that the nucleic acid of the OSV is RNA, even though in some cases small amounts of DNA may be associated with PCA extracts of viral RNA.

^{32}P-labeled virus

Nucleic acid extracted by the phenol method from purified suspensions of ^{32}P-labeled OSV was characterized. The nucleic acid type (RNA or DNA) was determined by subjecting viral nucleic acid to cesium sulfate gradient centrifugation, RNase treatment, and alkaline hydrolysis. Sedimentation coefficients were determined by glycerol gradient centrifugation, and anion exchange chromatography was used to determine nucleotide composition. Nucleic acid used for characterization was obtained from two separate experiments, designated here as Experiments 1

and 2.

The virus purification procedures used in Experiments 1 and 2 were exactly the same, and they are outlined in Table 3. ^{32}P-labeling and nucleic-acid-extraction methods differed slightly in the two experiments. In both, cell cultures were labeled with carrier-free ^{32}P-phosphoric acid shortly after virus was adsorbed to the monolayers. Cultures were labeled a second time at 24 hours PI in Experiment 1 and at 40 hours PI in Experiment 2.

Viral-infectivity and ^{32}P-radioactivity distributions after rate-zonal sucrose gradient centrification in Experiments 1 and 2 are presented in Figures 3a and 3b, respectively. The lowermost peaks of infectivity and radioactivity in each figure are located in a region with a sucrose density of approximately 1.20 g/cm^3, while the other predominant peaks are in a region of approximately 1.14 g/cm^3 sucrose. Good correspondence between ^{32}P-radioactivity and infectivity in the lower peaks was observed; however, these parameters are staggered in the peaks at a density of 1.14 g/cm^3.

A possible explanation of this latter observation is demonstrated in the photograph in Figure 4 of a sucrose gradient treated exactly as the gradients shown in Figure 3. The intensely opaque band located in the middle of the gradient represents the peaks shown in Figures 3a and 3b at a density of 1.14 g/cm^3. As mentioned earlier in connection with density determinations of OSV,

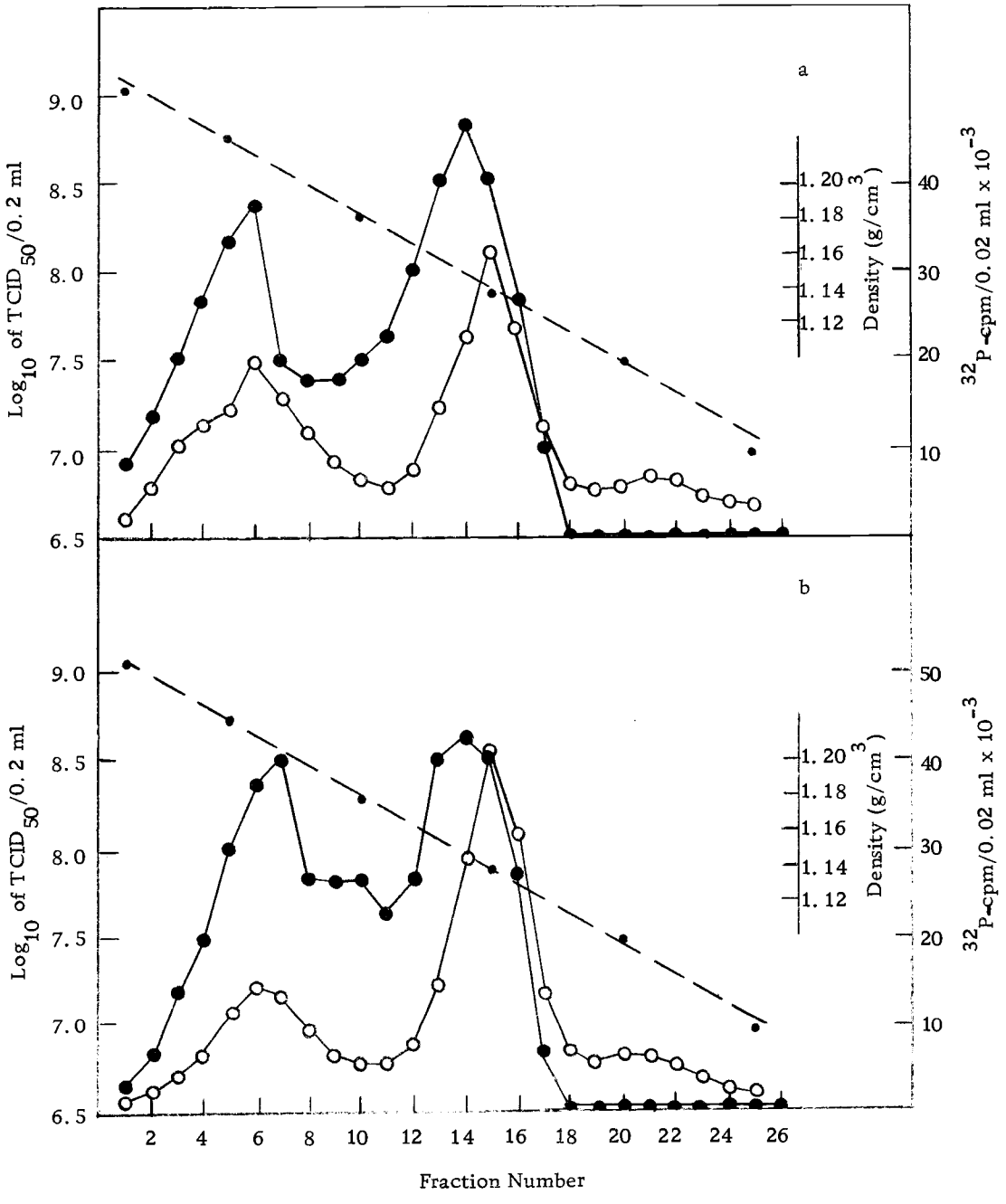
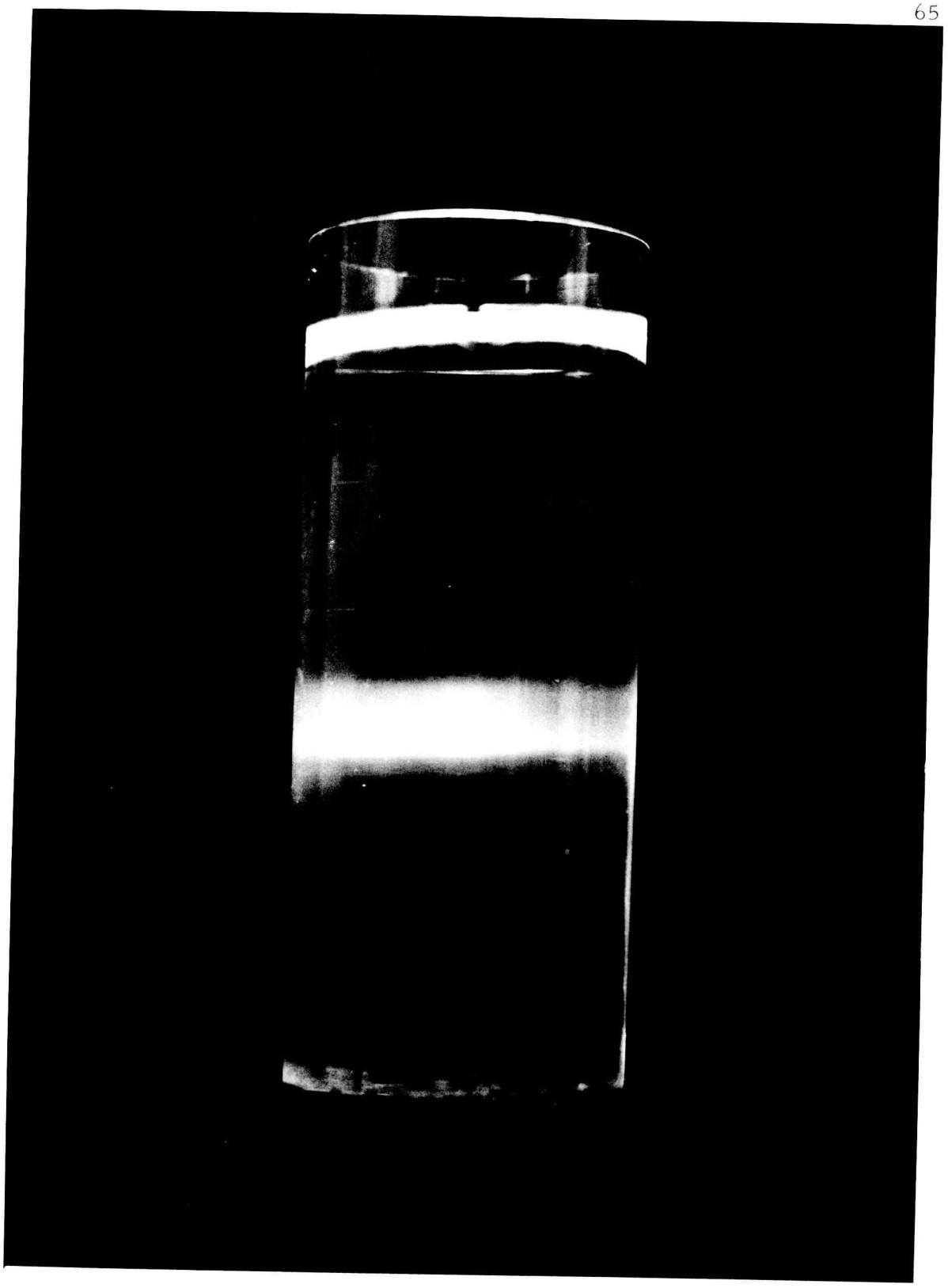


Figure 3. Centrifugation in a sucrose gradient of ^{32}P-labeled OSV. Ten to 55% (w/v) linear gradients were centrifuged at 42,000 x g for two hours, and 1.0 ml fractions were collected. Figures 3a and 3b represent two different experiments. ●, $\log_{10}$ of $TCID_{50}$; o, TCA-precipitable ^{32}P-counts; •, density.

Figure 4. The appearance of visible bands resulting from rate-zonal sucrose gradient centrifugation of a differentially centrifuged suspension of OSV. A 1 x 3-inch centrifuge tube containing a 10 to 55% sucrose gradient was photographed after centrifugation at 42,000 x g for 2 hours.



this band is composed of two closely associated bands. ^{32}P-radioactivity appears to be associated with both bands, but virus infectivity seems to correspond more to the lower band.

In addition to the double band described above, two other bands are visible in Figure 4. One band is one inch above and the other is one inch below the middle band. The diffuse lower band is in the same relative position as the infectivity and ^{32}P-radioactivity peaks located at a density of 1.20 g/cm^3 in Figures 3a and 3b. An infectivity peak at this density was also observed in sucrose gradients used to determine OSV density after centrifugation to equilibrium. However, considerably more infectivity was associated with this peak in Figures 3a and 3b than in density determination experiments.

The fractions containing the radioactivity peak located at a sucrose density of 1.14 g/cm^3, fractions 12 through 17 in Figures 3a and 3b, were pooled and used for nucleic acid extraction. In Experiment 1, the pooled fractions were phenol extracted once, and two phenol extractions were performed with the purified virus in Experiment 2. The precipitated viral nucleic acid in Experiment 2 was dissolved in 2.0 ml of TS-buffer, dispensed in 0.2-ml volumes, and frozen at -70°C. One 0.2-ml aliquot was diluted to 1.0 ml with TML-buffer, and absorbance at 230, 240, 250, 260, 270, and 280 mμ was determined. This absorption spectrum is

shown in Figure 5. Precipitated nucleic acid from Experiment 1 was dissolved in 1.52 ml of TML-buffer and immediately subjected to cesium sulfate gradient equilibrium centrifugation.

Most double-stranded DNA and single-stranded RNA have been found to band at cesium sulfate densities of approximately 1.42 and 1.64 g/cm^3, respectively, when centrifuged to equilibrium in cesium sulfate gradients (Szybalski, 1968). The distribution of ^{32}P-labeled OSV nucleic acid in equilibrium-centrifuged cesium sulfate gradients was determined in order to provide additional evidence to identify the type of viral nucleic acid.

Preliminary experiments were performed to find the optimum volumes of saturated cesium sulfate and sample necessary for banding both RNA and DNA in one gradient. The distribution of ^{14}C-labeled chick myeloblast nucleic acid (specific activity, 10,000 cpm/μg) in a cesium sulfate gradient is presented in Figure 6. This gradient contained the same volumes of cesium sulfate and sample, and was centrifuged in the same manner as the cesium sulfate gradients containing OSV nucleic acid described below. Peaks of ^{14}C-radioactivity were located at cesium sulfate densities of 1.65 and 1.42 g/cm^3, corresponding to RNA and DNA, respectively.

The distribution of ^{32}P-labeled OSV nucleic acid in two cesium sulfate gradients and a summary of the methodology are

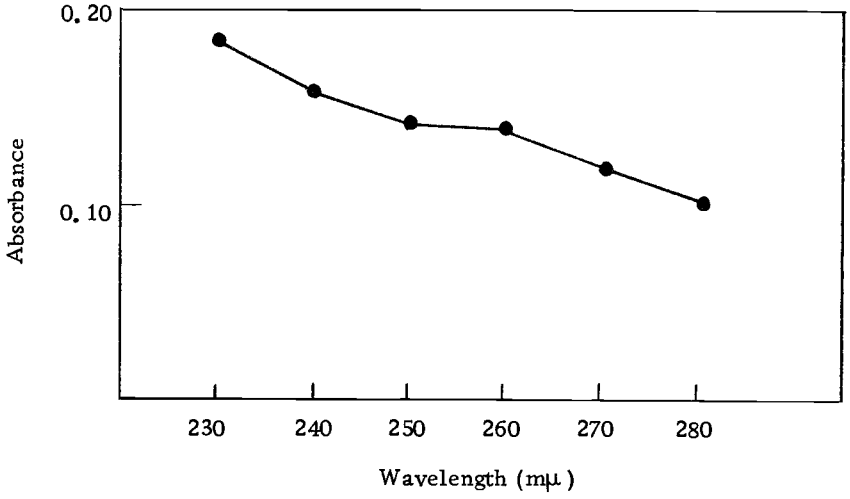


Figure 5. Ultraviolet spectrum of a nucleic acid solution phenol-extracted from a purified suspension of ^{32}P-labeled OSV.

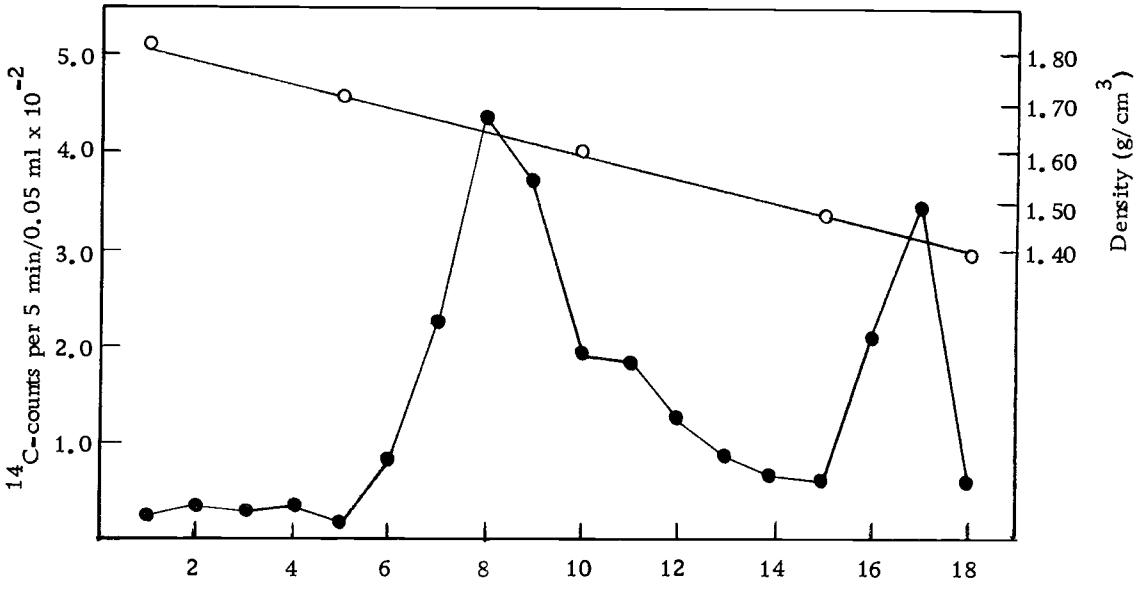


Figure 6. Equilibrium centrifugation in a cesium sulfate gradient of ^{14}C-labeled chick myeloblast nucleic acid. ●, ^{14}C-counts; o, density. (Figure 7 contains methodology.)

given in Figure 7. The nucleic acid solutions in the gradients shown in Figures 7a and 7b were from Experiments 1 and 2, respectively. A single radioactivity peak was observed in both gradients at cesium sulfate densities of 1.58 (Fig. 7a) and 1.59 g/cm^3 (Fig. 7b). Each peak had a shoulder at approximately 1.61 g/cm^3.

In addition to measuring the ^{32}P-radioactivity of each fraction from cesium sulfate gradients, the absorbance at 260 and 280 mμ was determined. The values for absorbance at 260 mμ are shown in Figure 7. Fractions 12 and 11 in Figures 7a and 7b, respectively, contained the highest absorbance values in each gradient. Fraction 12 had a 260/280 mμ ratio of 1.3 and the 260/280 mμ ratio of fraction 11 was 1.1.

A 0.2-ml sample of ^{32}P-labeled OSV nucleic acid from Experiment 2, containing 16,000 cpm, was mixed with ^{14}C-labeled chick myeloblast nucleic acid solution having 20,000 cpm (specific activity, 10,000 cpm/μg) to give a final volume of 0.5 ml in TSE-buffer. This volume was layered onto a 5-ml, 10 to 30% glycerol gradient. After centrifugation at 179,000 x g for 2.5 hours, 13-drop fractions were collected, the bottom of the nitrocellulose centrifuge tube was rinsed with 1.0 ml of TSE-buffer, and each fraction was diluted to 1.0 ml with TSE-buffer. A 0.5-ml aliquot was removed from each diluted fraction. These aliquots were plated, and ^{32}P and ^{14}C-radioactivity was determined. The remaining 0.5-ml volume of

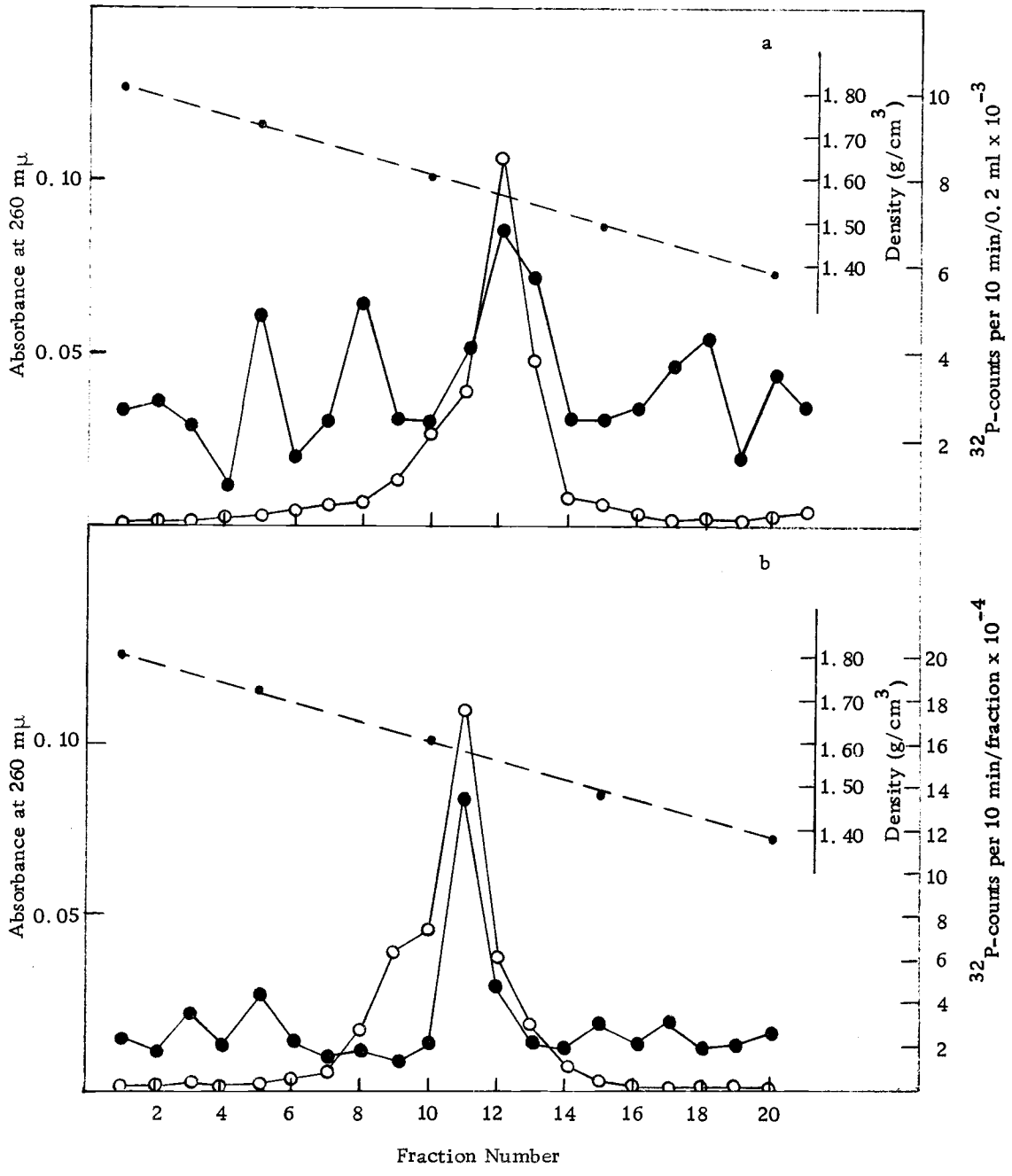


Figure 7. Equilibrium centrifugation in a cesium sulfate gradient of phenol-extracted ^{32}P-labeled OSV nucleic acid. Gradients contained 1.52 ml of sample and 1.98 ml of saturated cesium sulfate, with 2.0 ml of mineral oil layered on top. Gradients were centrifuged at 130,000 x g for 60 hours at 22°C. Nucleic acid in gradients shown in Figures 7a and 7b was obtained from virus banded in sucrose gradients shown in Figures 3a and 3b, respectively. •, absorbance at 260 mμ; o, TCA-precipitable ^{32}P-counts; •, density.

each fraction was treated with RNase by adding 0.1 ml of a 100 µg/ml RNase solution and incubating at 30°C for 40 min. The amount of TCA-precipitable radioactivity in the RNase-treated portion of the fractions was determined in the same manner as the untreated portions.

The results of glycerol gradient centrifugation are presented in Figure 8. ^{14}C-radioactivity in the untreated fractions was distributed into three peaks. The 18S and 28S peaks represented myeloblast cell ribosomal RNA (Watson, 1970). Using the location of these two peaks as references, the sedimentation coefficients of the ^{32}P-labeled OSVnucleic acid distribution were estimated. ^{32}P-radioactivity was dispersed over the entire gradient, with a single major peak located in the 26S region and a pronounced shoulder at approximately 37S.

TCA-precipitable radioactivity in the RNase-treated portion of the glycerol gradient fractions was greatly reduced. All of the ^{32}P-radioactivity from OSV nucleic acid was sensitive to RNase, indicating that the OSV nucleic acid was RNA. A similar effect was observed with ^{14}C-labeled myeloblast cell nucleic acid, except a definite peak of RNase-resistant radioactivity was located at a 3S position. Since a previous experiment demonstrated the presence of DNA in the ^{14}C-myeloblast nucleic-acid preparation (see Figure 6), the RNase-resistant peak in Figure 8 represented

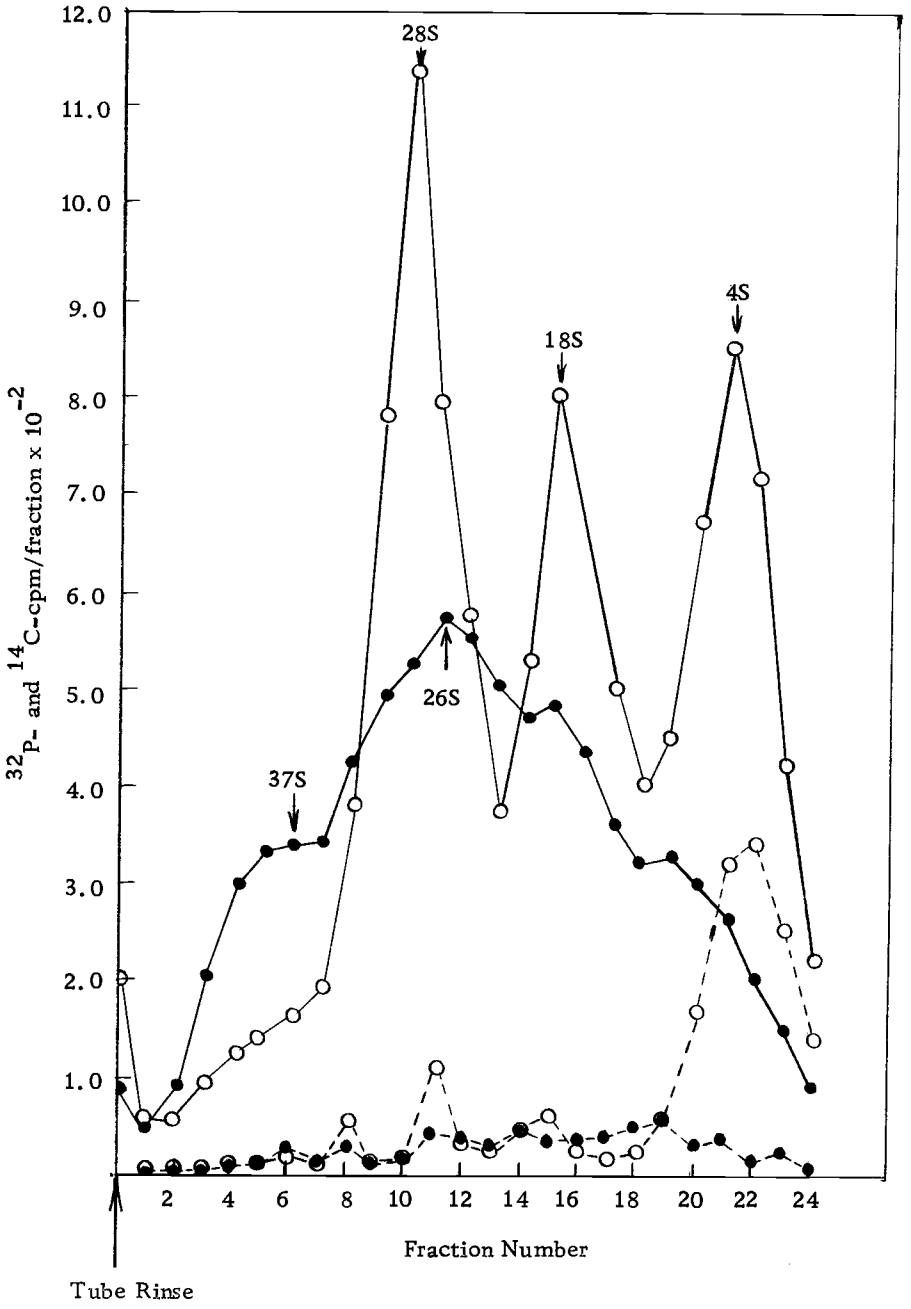


Figure 8. Centrifugation in a glycerol gradient of phenol-extracted ^{32}P-labeled OSV nucleic acid and ^{14}C-labeled chick myeloblast cell nucleic acid (used as a marker). A linear 10 to 30% (w/v) glycerol gradient containing 0.01M Tris buffer, 0.1M NaCl, and 0.001M EDTA was centrifuged at 179,000 x g for 2.5 hours at 3°C. One half of each fraction was treated with 17 μg/ml RNase. o—o, TCA-precipitable ^{14}C-counts; •—•, TCA precipitable ^{32}P-counts; o---o, TCA-precipitable ^{14}C-counts (RNase-treated); •---•, TCA-precipitable ^{32}P-counts (RNase-treated).

^{14}C-labeled DNA.

OSV ^{32}P-labeled nucleic acid banded in the cesium sulfate gradient described in Figure 7b was used for nucleotide composition analyses. Fraction 12 from the gradient was divided into three aliquots, and each aliquot was combined with 1.5 mg of unlabeled bulk E. coli RNA. The RNA in each sample was precipitated with 10% TCA, collected on a membrane filter, and hydrolyzed in 3.0 ml of 0.3N KOH at 37°C for 18 hours. The nucleotides in the hydrolyzed samples were separated by anion exchange chromatography, and the percentage distribution of molarity and ^{32}P-radioactivity in the four nucleotides was determined.

The anion exchange chromatography technique and the results of one analysis are described in Figure 9. A hydrolyzed RNA sample and appropriate eluting solvents were passed through a Dowex 1-x-8, 100-200 mesh, 0.87 x 5.0 cm column, from which 5-ml fractions were collected. The absorbance at 260 mμ and ^{32}P-radioactivity of each fraction were determined. Absorbance values were used to follow the separation of the nucleotides and to calculate the nucleotide composition of the bulk E. coli RNA (see Table 6).

By plotting the absorbance at 260 mμ versus fraction number, the distribution of the unlabeled nucleotides was obtained. The total absorbance in each absorption peak was totalled, and the

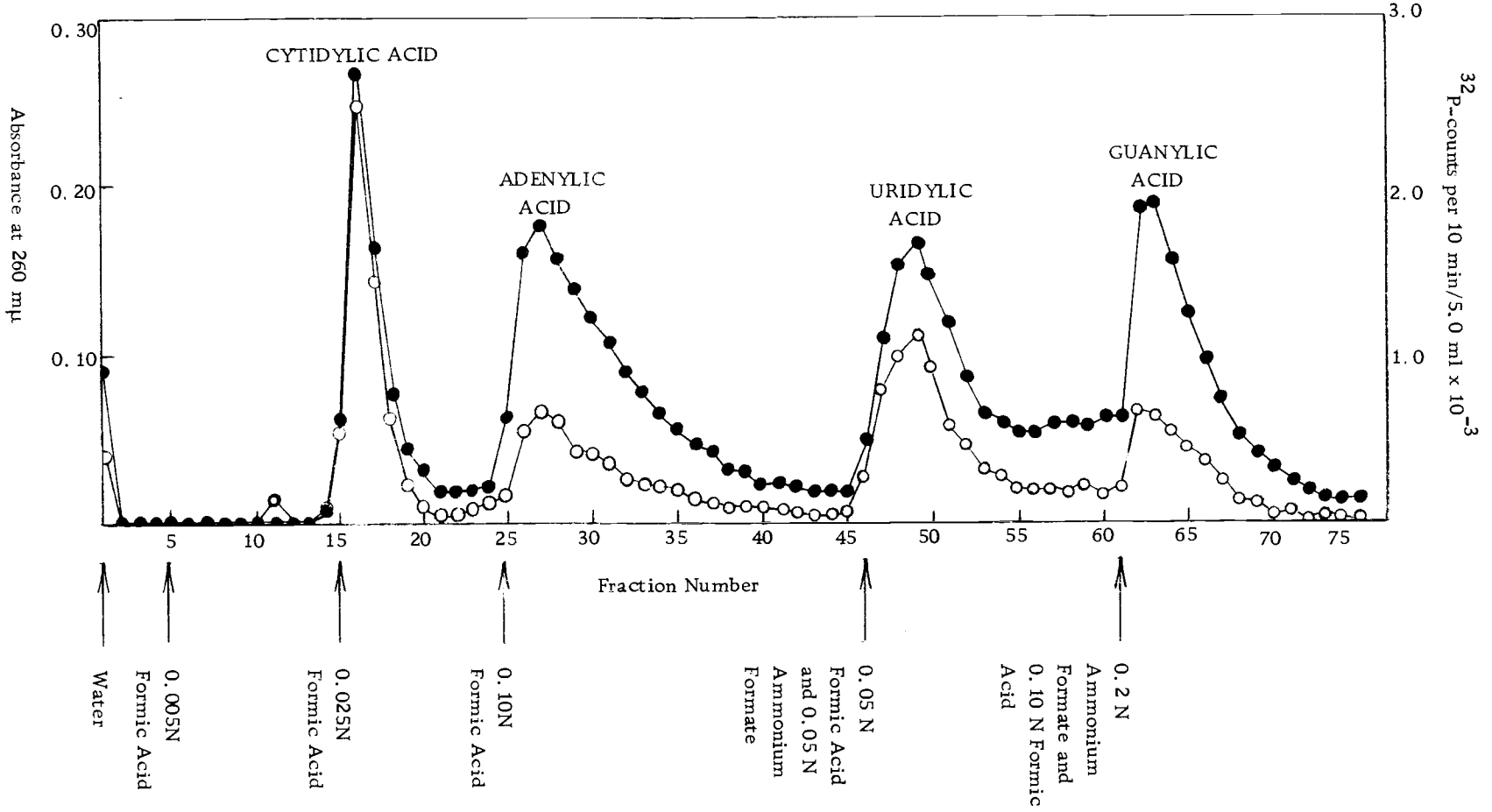


Figure 9. Anion exchange chromatography of OSV nucleic acid from fraction 11 of the cesium sulfate gradient shown in Figure 7b. Three ml of a solution containing OSV nucleic acid and unlabeled bulk E. coli RNA were loaded on a 0.87 x 5.0 cm column of Dowex 1-x-8 converted to the formate form. The fractions at which each eluting solution was added are indicated by arrows at the bottom of the figure. o, ^{32}P-counts; •, absorbance at 260 mμ

Table 6. Calculation of percent ^{32}P radioactivity and percent molarity from the distribution of radioactivity and absorbance resulting from anion exchange chromatography presented in Figure 9.

Nucleotide	Fractions in Peak		Total Values		*E. coli* RNA		OSV RNA
	A_{260}	^{32}P-cpm	A_{260}	^{32}P-cpm	Molarity x 10^2	% Molarity	% ^{32}P-cpm
Cytidylic acid	15-20	14-20	0.617	540	0.995	23.5	26.0
Adenylic acid	23-42	23-41	1.483	479	1.022	24.3	23.0
Uridylic acid	46-54	46-54	0.898	577	0.898	21.4	27.8
Guanylic acid	54-73	54-71	1.499	481	1.290	30.7	23.2

molarity of each corresponding nucleotide was calculated by dividing the absorbance value by the appropriate extinction coefficient for 260 mμ at pH 2.0. Extinction coefficients employed and the order in which the nucleotides eluted off the column were as follows: cytidylic acid (C), 6.2×10^{-3}; adenylic acid (A), 14.5×10^{-3}; uridylic acid (U), 10.0×10^{-3}; and guanylic acid (G), 11.6×10^{-3} (Schwarz Bio Research, Inc. Catalog, 1969). The molarities of the nucleotides were totalled and the percent molarity of each was calculated (see Table 6). Cut-off points between absorption peaks were adjusted until the percent molarity for each nucleotide corresponded most closely to the known values for bulk E. coli RNA (C, 24.3%; A, 25.0%; U, 19.7%; and G, 31.0% (Hayashi and Spiegelman, 1961)).

Using the cut-off points established with bulk E. coli RNA, the ^{32}P-cpm in the four peaks representing each nucleotide was totalled, and the percent radioactivity in the nucleotides was calculated. Table 7 contains the results of three analyses. The A+U/G+C ratio for OSV RNA was 1.03.

The nucleotide composition of OSV RNA shown in Table 6 was determined from three analyses of one batch of extracted viral RNA. Multiple anion-exchange-chromatograph analyses should be performed on additional samples of OSV RNA before the nucleotide composition values given in Table 6 can be regarded as established.

Table 7. The results of nucleotide composition determinations using anion exchange chromatography.

	Preparation Number:							
	One		Two		Three		Average Values ± 1 S. D.	
Nucleotide	% ^{32}P[a]	Mole %[b]	% ^{32}P	Mole %	% ^{32}P	Mole %	% ^{32}P	Mole %
Cytidylic acid	26.3	23.5	25.1	23.1	26.0	23.5	25.8±0.6	23.4±0.2
Adenylic acid	22.2	26.0	23.8	26.3	23.0	24.3	23.0±0.8	25.5±1.1
Uridylic acid	28.2	20.2	27.1	19.6	27.8	21.4	27.7±0.6	20.4±0.9
Guanylic acid	23.2	30.0	23.9	31.1	23.2	30.7	23.4±0.4	20.6±0.6

[a] % ^{32}P refers to the percent ^{32}P radioactivity of each nucleotide in hydrolyzed OSV RNA.

[b] Mole % refers to the percent molarity of each nucleotide in hydrolyzed bulk E. coli RNA.

Cross Plaque Neutralization Tests

Antigenic relationships between OSV, IHN virus, and SRCD virus were investigated using cross plaque neutralization tests. Rabbit antisera against OSV, SRCD virus and IHN virus were produced by immunizing rabbits with partially purified suspensions of each virus propagated in cell line SSE-30. Tests were performed with stocks of OSV, SRCD virus, and IHN virus propagated in SSE-30 cell cultures and frozen at -60°C in complete MEM.

Injection schedules and the infectivity of virus suspensions used to immunize rabbits are listed in Table 8. In general, an injection schedule consisted of two series of injections, about 14 days apart. The first series involved a 1.0-ml intraveneous (I. V.) injection of undiluted virus suspension and two 1.0-ml intramuscular (I. M.) injections of a sterile emulsion composed of equal parts virus suspension and Complete Freund's adjuvant. The second series of injections were the same as the first, except no I. V. injection was given.

Rabbit serum used in plaque neutralization tests was sterilized by vacuum Millipore filtration and stored at 4°C. Shortly before each test, serum was heat treated at 56°C for 30 min. In the cross plaque neutralization tests described below, immune sera were collected approximately three weeks after the second injection series.

Table 8. Description of technique used for rabbit immunization with OSV, IHN virus, and SRCD virus suspensions.

Antigen	Injection Series	Infectivity ($TCID_{50}$/ml) in the Undiluted Antigen Suspension*	Days between Last Injection and Preparation of Test Antiserum
OSV	First	1.1×10^9	--
	Second	1.6×10^9	25
SRCD	First	1.6×10^9	--
	Second	2.0×10^9	19
IHN	First	1.6×10^9	--
	Second	5.0×10^8	18

*No. of $TCID_{50}$/ml of virus suspension before emulsifying with an equal volume of adjuvant.

Two cross plaque neutralization tests were performed with each antiserum versus the three viruses. The test procedure involved the following steps: (1) rabbit immune and normal serum were diluted with MEM-diluent (MEM with 0.4% BSA) to give five two-fold antiserum dilutions and one normal serum dilution; (2) each serum dilution was dispensed into three tubes, 1.0 ml per tube, yielding three identical sets of dilutions; (3) each stock virus was diluted with MEM-diluent to give an estimated 100 pfu/0.15 ml;

(4) 1.0-ml volumes of this suspension were distributed to one set of serum dilutions, and the tubes were incubated at 18°C for 60 min; (5) residual virus in each serum-virus mixture was assayed in triplicate by the monolayer plaque assay technique using 2-oz prescription bottles with confluent monolayers of cell line CHSE-214 (see Methods and Materials, Infectivity Assays).

The chinook salmon cell line, CHSE-214, was used for plaque neutralization tests in an effort to minimize possible cytopathic effects caused by host-cell specific rabbit antibodies. Virus suspensions used to immunize rabbits were propagated in the sockeye salmon cell line, SSE-30, and probably contained some host-cell material. Monolayers of cell line CHSE-214 treated with rabbit antiserum against OSV showed no signs of cellular degeneration when examined microscopically. In addition, OSV, IHN virus, and SRCD virus all formed distinct, 1- to 2-mm plaques in monolayers of CHSE-214 after incubation at 18°C for five days.

The results of two sets of two cross plaque neutralization tests with OSV and SRCD rabbit antiserum versus each of the three viruses are presented in Tables 9 and 10. The Log_{10} of each percent plaque neutralization value from this table is plotted versus the corresponding antiserum dilution in Figures 10 and 11. Each set of points seemed to be in a linear arrangement, and best-fitting dose-response curves were drawn for each set. The antiserum dilution

Table 9. Cross-plaque neutralization tests with OSV rabbit antiserum versus OSV, IHN virus, and SRCD virus.

Virus	Reciprocal of Antiserum Dilution	Experiment I Plaque Counts Per Bottle	Experiment I Plaque Counts Ave.	Experiment I % Neut.	Experiment II Plaque Counts Per Bottle	Experiment II Plaque Counts Ave.	Experiment II % Neut.
OSV	200	37, 36, 37	37	68	23, 19, 23	22	76
	400	63, 72, 72	69	40	55, 56, 52	54	40
	800	87, 86, 77	83	27	72, 64, 69	68	24
	1600	98, 81, 97	92	19	86, 74, 71	77	15
	3200	101, 93, 101	99	13	101, 93, 87	94	--
	Controls*	107, 118, 119	114	--	99, 89, 82	90	--
IHN	200	30, 37, 35	34	81	10, 10, 8	10	89
	400	80, 96, 97	91	50	44, 36, 40	40	55
	800	155, 138, 170	154	16	50, 50, 66	56	36
	1600	167, 147, 167	161	12	68, 71, 66	68	23
	3200	169, 178, 179	175	4	71, 78, 80	76	14
	Controls*	177, 182, 191	183	--	84, 92, 88	88	--
SRCD	200	6, 7, 6	7	96	1, 1, 1	1	99
	400	63, 67, 63	64	62	16, 21, 20	19	80
	800	119, 104, 129	117	31	52, 46, 49	49	49
	1600	144, 150, 123	139	18	68, 67, 61	65	32
	3200	189, 165, 161	172	--	79, 72, 85	79	18
	Controls*	156, 173, 177	169	--	90, 98, 101	96	--

*Controls were virus suspension mixed 1:1 with diluent containing a 1/100 dilution of normal rabbit serum.

Table 10. Cross-plaque neutralization tests with SRCD virus rabbit antiserum versus OSV, IHN virus, and SRCD virus.

Virus	Reciprocal of Antiserum Dilution	Experiment I Plaque Counts Per Bottle	Experiment I Plaque Counts Ave.	Experiment I % Neut.	Experiment II Plaque Counts Per Bottle	Experiment II Plaque Counts Ave.	Experiment II % Neut.
SRCD	1000	6, 6, 5	6	92	7, 8, 12	9	92
	2000	28, 24, 29	27	66	32, 31, 29	31	73
	4000	41, 39, 48	43	46	59, 69, 73	66	43
	8000	47, 51, 50	49	38	75, 91, 95	87	26
	16000	57, 61, 51	56	29	111, 93, 92	99	15
	Controls*	86, 77, 74	79	--	123, 107, 120	117	--
OSV	1000	19, 23, 20	21	75	40, 33, 32	35	63
	2000	46, 42, 39	42	50	58, 47, 59	55	42
	4000	63, 56, 51	57	31	68, 84, 75	76	20
	8000	70, 59, 60	63	15	78, 77, 94	83	13
	16000	75, 72, 83	77	8	106, 93, 91	97	--
	Controls*	75, 92, 85	84	--	100, 84, 101	95	--
IHN	1000	22, 19, 23	21	73	35, 44, 38	39	53
	2000	49, 53, 44	49	37	55, 43, 53	50	40
	4000	55, 64, 61	60	23	73, 61, 69	67	19
	8000	78, 79, 65	74	5	71, 68, 80	73	12
	16000	72, 84, 88	81	--	87, 76, 84	82	--
	Controls*	86, 72, 76	78	--	80, 77, 92	83	--

*Controls were virus suspension mixed 1:1 with diluent containing a 1/100 dilution of normal rabbit serum.

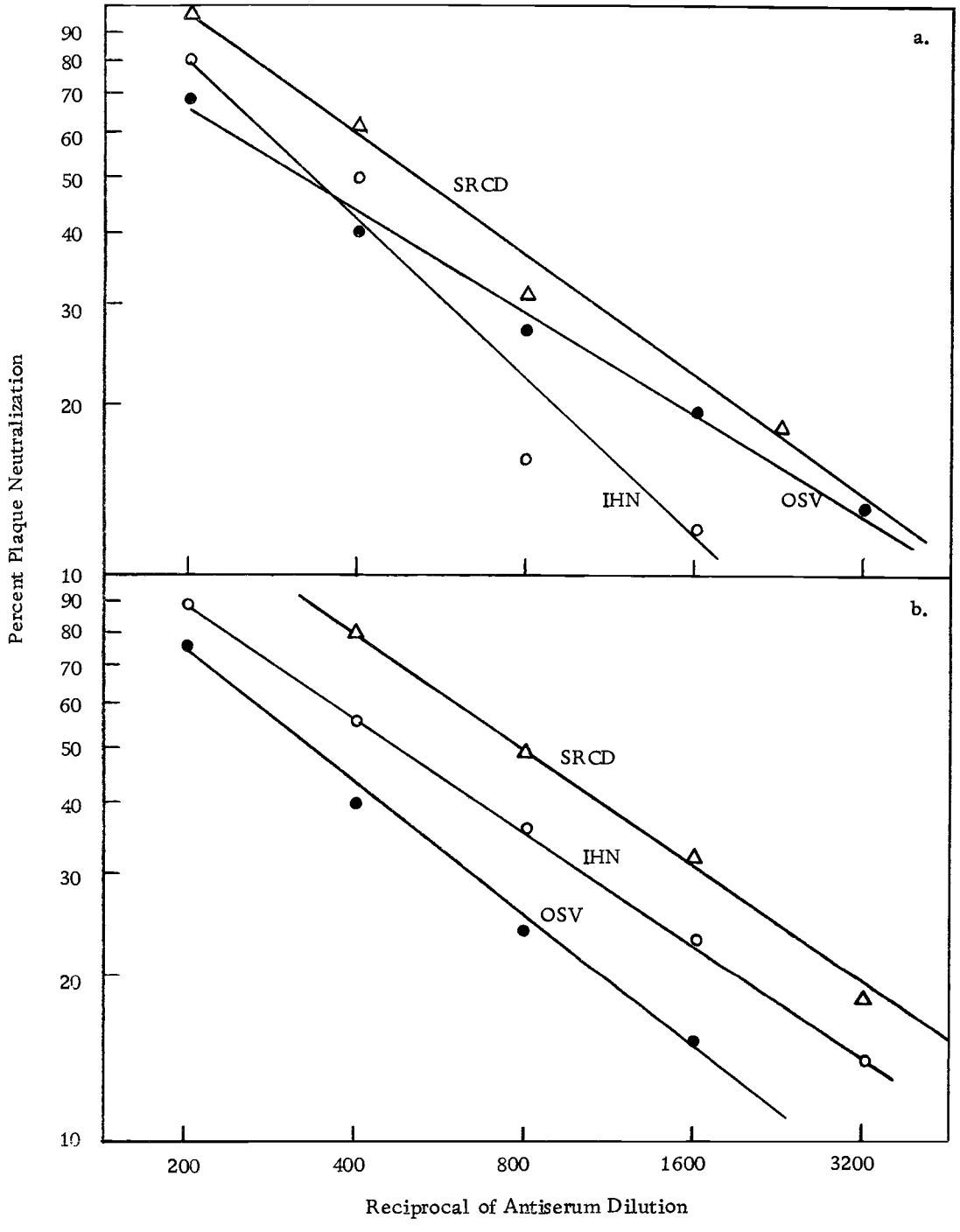


Figure 10. Cross-plaque neutralization tests with OSV rabbit antiserum tested against OSV, IHN virus, and SRCD virus. Dose response curves from data in Table 9. a., Experiment I; b., Experiment II. Δ, SRCD virus; •, OSV; o IHN virus.

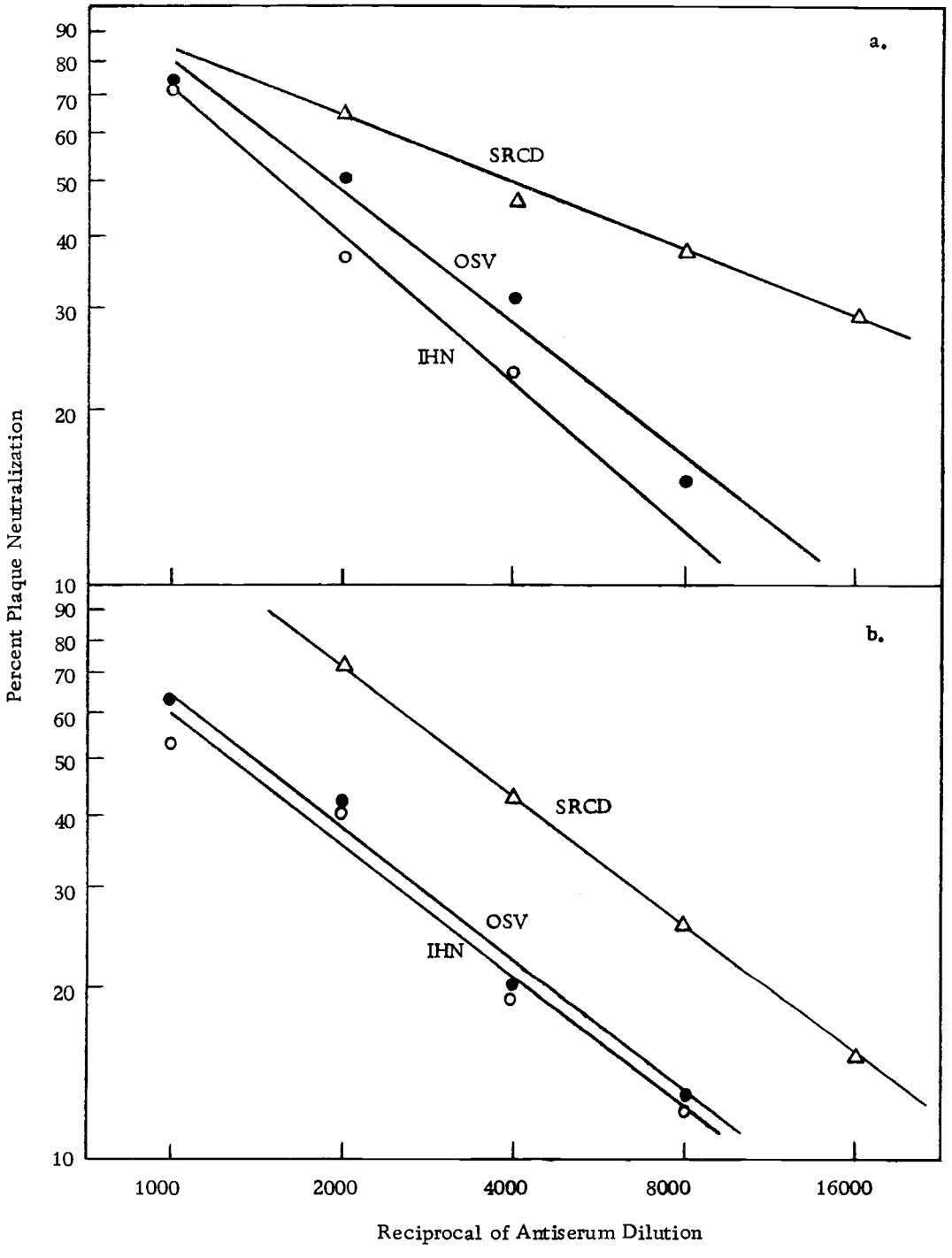


Figure 11. Cross-plaque neutralization tests with SRCD virus rabbit antiserum tested against OSV, IHN virus, and SRCD virus. Dose response curves from data in Table 10. a., Experiment I; b., Experiment II. △, SRCD virus; •, OSV; o, IHN virus.

corresponding to the point at which each curve intersected the line representing 50% plaque neutralization was considered the end point. Table 11 contains the 50% plaque neutralization end points for each of the four cross plaque neutralization tests.

The data in Table 11 indicate that OSV and IHN virus are antigenically very closely related. The SRCD virus appears to be antigenically related to the other two viruses, although the end points for SRCD virus in all four tests were from 1.6 to 2.5 times higher than the end points for OSV and IHN virus.

Apparent antigenic differences between the SRCD virus and the Oregon sockeye salmon and IHN viruses may be influenced by the host specificity of SRCD virus for cell line CHSE-214. The effect of host specificity was indicated in cross plaque neutralization tests with OSV antiserum. In both tests, SRCD virus was neutralized to a greater degree than the homologous virus.

Two cross plaque neutralization tests with IHN rabbit immune serum versus the three viruses were also performed. These results are displayed in Table 12. Each of the plaque neutralization tests versus each virus in both cross-plaque neutralization tests yielded only two statistically significant percent plaque neutralization values. Fifty percent plaque neutralization end points determined from dose response curves drawn for each set of two points were probably not as statistically reliable as the end points obtained from three or more

Table 11. Cross-plaque neutralization tests with OSV, SRCD virus, and IHN virus antisera versus OSV, SRCD virus, and IHN virus. Fifty percent neutralization end points (50% NEP)[a] determined from data in Tables 9, 10 and 12.

	OSV[b]		SRCD Virus[b]		IHN Virus[c]	
Virus	Expt. I 50% NEP	Expt. II 50% NEP	Expt. I 50% NEP	Expt. II 50% NEP	Expt. I 50% NEP	Expt. II 50% NEP
SRCD	540	780	3900	3400	560	346
OSV	330	350	1900	1460	140	144
IHN	340	480	1600	1330	152	164

[a] Fifty percent neutralization end point (50% NEP) represents the reciprocal of the antiserum dilution causing a 50% reduction in plaque numbers.

[b] In tests where OSV and SRCD virus antisera were employed, 50% NEP values were determined from dose response curves drawn for three or more points.

[c] In tests where IHN virus antiserum was employed, 50% NEP values were determined from dose response curves drawn for two or more points.

Table 12. Cross-plaque neutralization tests with IHN virus rabbit antiserum versus OSV, IHN virus, and SRCD virus.

Virus	Reciprocal of Antiserum Dilution	Experiment I Plaque Counts Per Bottle	Experiment I Plaque Counts Ave.	Experiment I % Neut.	Experiment II Plaque Counts Per Bottle	Experiment II Plaque Counts Ave.	Experiment II % Neut.
IHN	40	0, 0, 3	1	--	0, 0, 0	0	--
	80	11, 13, 10	11	95	7, 8, 8	8	93
	160	113, 114, 120	116	47	45, 45, 56	49	57
	320	178, 194, 191	187	14	90, 94, 104	96	16
	640	228, 249, 243	240	--	121, 109, 117	116	--
	Controls*	199, 226, 227	217	--	115, 122, 105	114	--
OSV	40	1, 4, 5	3	--	0, 0, 0	0	--
	80	15, 22, 18	18	92	10, 10, 11	10	91
	160	149, 121, 118	129	41	58, 65, 62	62	45
	320	209, 191, 176	192	12	105, 105, 89	100	11
	640	185, 226, 218	210	4	121, 107, 101	110	3
	Controls*	227, 205, 224	218	--	110, 110, 120	113	--
SRCD	40	0, 0, 0	0	--	0, 0, 0	0	--
	80	0, 0, 0	0	--	0, 0, 0	0	--
	160	1, 2, 0	1	--	2, 1, 2	2	--
	320	39, 41, 33	38	68	27, 29, 31	29	54
	640	66, 64, 68	66	45	54, 57, 46	52	17
	Controls*	120, 129, 109	119	--	61, 67, 61	63	--

*Controls were virus suspension mixed 1:1 with diluent containing a 1/20 dilution of normal rabbit serum.

points in tests with OSV and SRCD antisera. Nevertheless, the 50% plaque neutralization end points obtained with IHN antiserum are presented in Table 11 in order to support the data obtained with the other two antisera.

Fifty percent neutralization end points determined for each type of rabbit immune serum were proportional to the infectivity of the virus suspensions used for rabbit immunization. Rabbits immunized with suspensions of SRCD virus, containing a total of 3.6×10^9 $TCID_{50}$/ml, yielded antiserum with end points between 1/1330 and 1/3900. Antiserum against OSV had 50% neutralization end points from 1/330 to 1/780 and was obtained from rabbits injected with suspensions containing a total of 2.7×10^9 $TCID_{50}$/ml. The lowest end points (1/140 to 1/560) were determined for antiserum from rabbits receiving suspensions of IHN virus containing a total of 2.1×10^9 $TCID_{50}$/ml.

Electron Microscopy

Partially purified suspensions of OSV were examined with a Philips EM-300 electron microscope. Virus suspensions were purified by differential centrifugation and prepared for electron microscope examination by two different methods. One technique consisted of fixing partially purified virus with 0.5% glutaraldehyde for 20 min at $4^{\circ}C$. Drops of a fixed virus suspension were allowed

to remain on Formvar-coated copper grids for five min, and excess fluid was removed with filter paper. Droplets of 1.0% PTA, pH 6.5, were applied to the grids in the same manner and removed after about five seconds. Polystyrene latex (PSL) spheres (diameter 264 ± 6 mμ, the Dow Chemical Co.) suspended in double distilled water were sprayed onto grids with a glass nebulizer.

In the other method, partially purified unfixed virus suspended in 1% ammonium acetate was mixed one to one with either 1.0% or 2.0% PTA, pH 6.5. This suspension was sprayed onto carbon-stabilized, Formvar-coated copper grids.

Figures 12, 13, and 14 (1) are electron micrographs of the same glutaraldehyde-fixed virus suspension. Figures 14(2), (3) and (4) are micrographs of representative fields demonstrating the particles observed in unfixed virus preparations. Direct magnification values used to calculate the magnifications given for these figures were obtained from the microscope manufacturer's manual. The diameters of PSL spheres, included in some of the micrographs as internal standards, were determined using these magnifications; the diameters were within the size range specified for that lot of spheres.

Although virus suspensions used in fixed and unfixed preparations contained approximately 10^9 $TCID_{50}$/ml, electron microscope examination of these suspensions revealed a variety of particle

Figure 12. An electron micrograph of a differentially centrifuged and glutaraldehyde fixed suspension of OSV, negatively stained with 1% PTA. The dense sphere in the center is a PSL sphere, 264 ± 6 mμ in diameter. Five typical appearing, bullet-shaped particles are present. A short particle with visible striations is located in the lower, right corner. Magnification, 1.27×10^5 X.

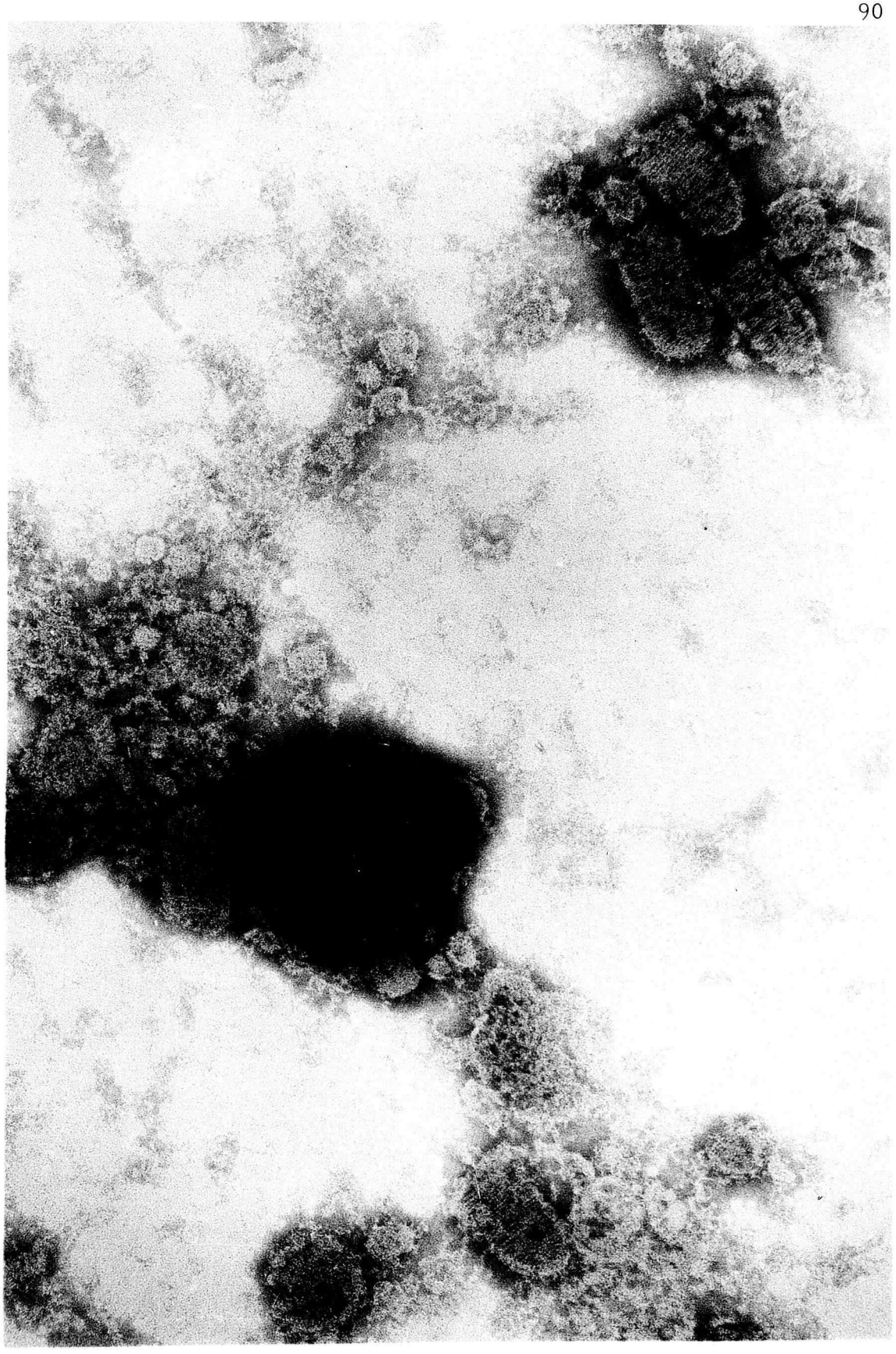


Figure 13. An electron micrograph of the same virus preparation shown in Figure 12. Twenty-seven well defined, bullet-shaped particles, and three truncated forms are present. Magnification, 3.99×10^4 X.

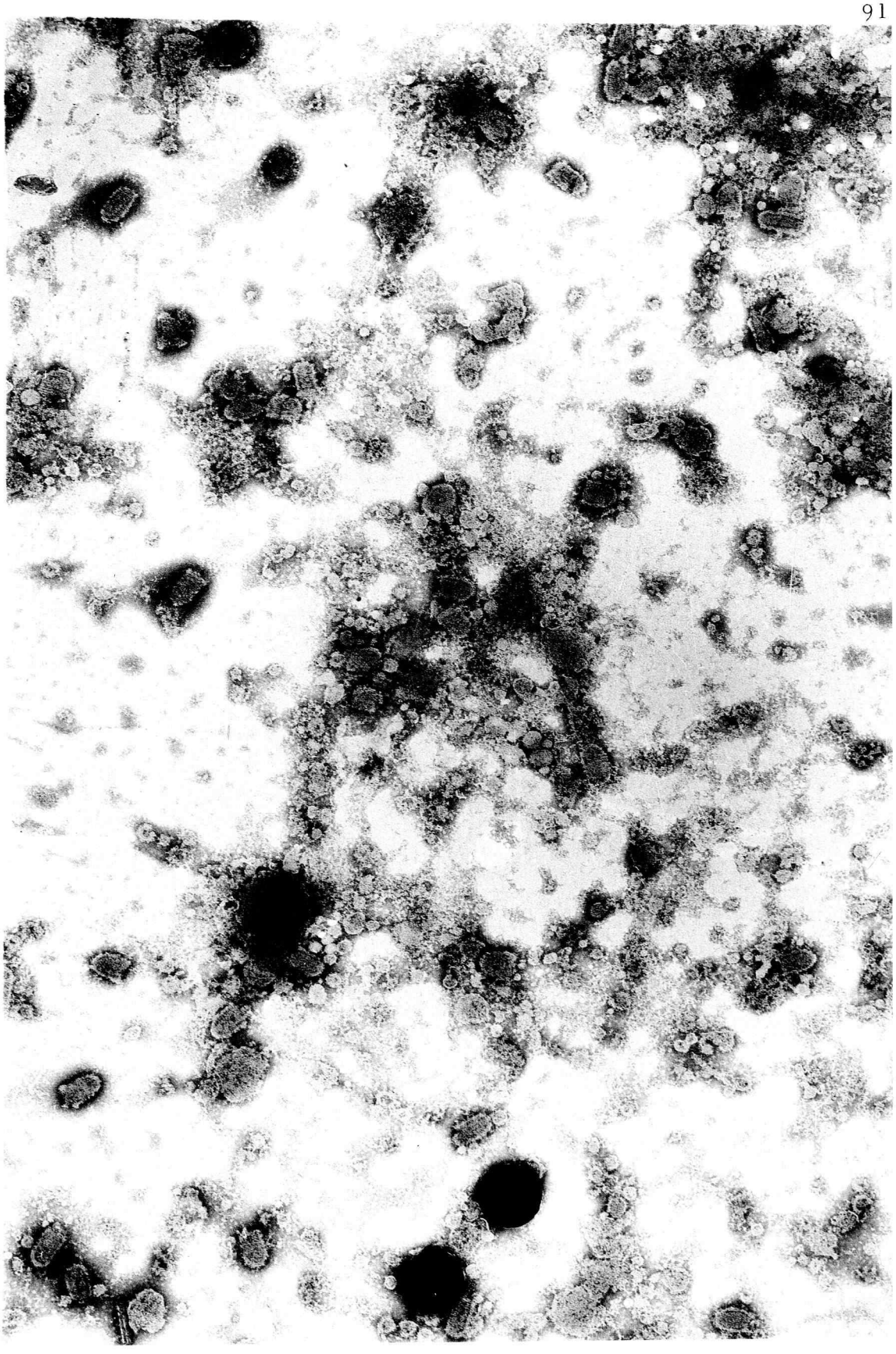
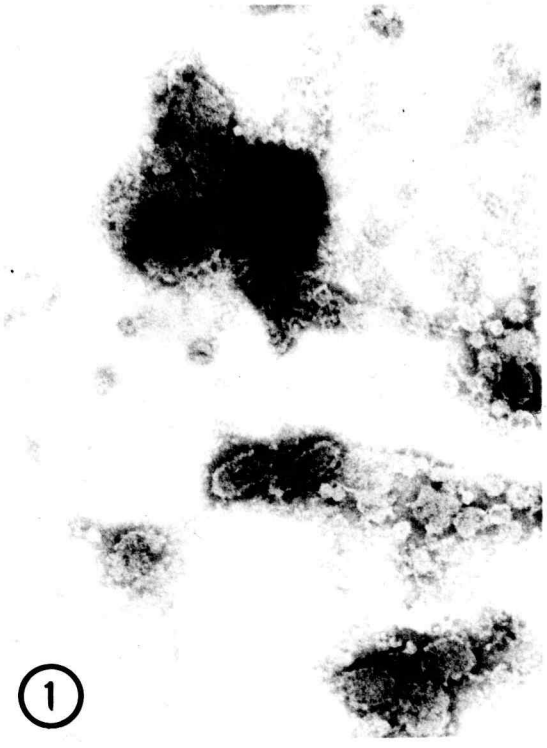


Figure 14. Electron micrographs of glutaraldehyde-fixed and unfixed suspensions of OSV.

(1) A micrograph of the same virus preparation shown in Figures 12 and 13. One of the two particles in the center appears to be rounded at both ends and completely surrounded at both ends by an outer coat. Magnification, 4.45×10^4 X.

(2), (3), and (4) Representative fields of an unfixed OSV suspension which was partially purified by differential centrifugation, and was suspended in 1% ammonium acetate. This suspension was mixed 1:1 with 2% PTA, and sprayed onto carbon-stabilized, Formvar-coated, copper grids with a nebulizer. Magnifications, 5.3×10^4 X.



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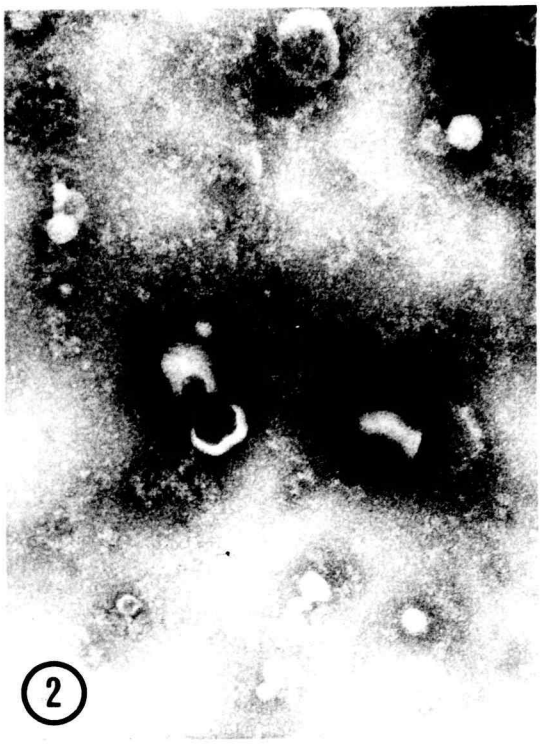

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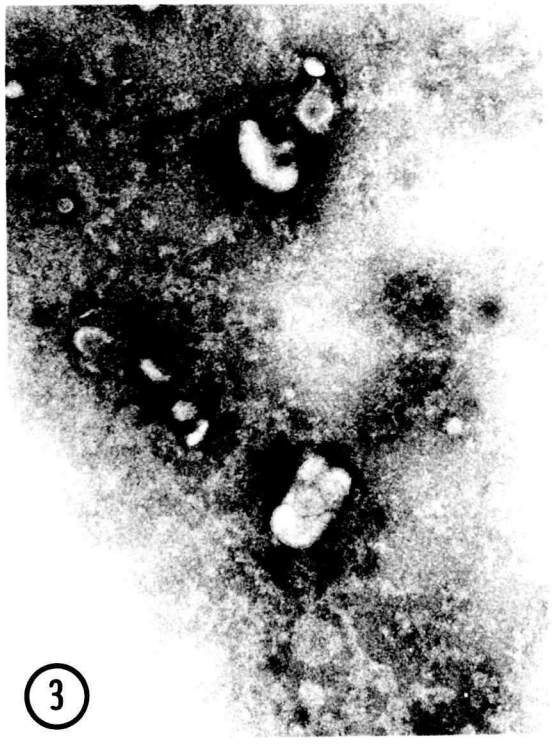

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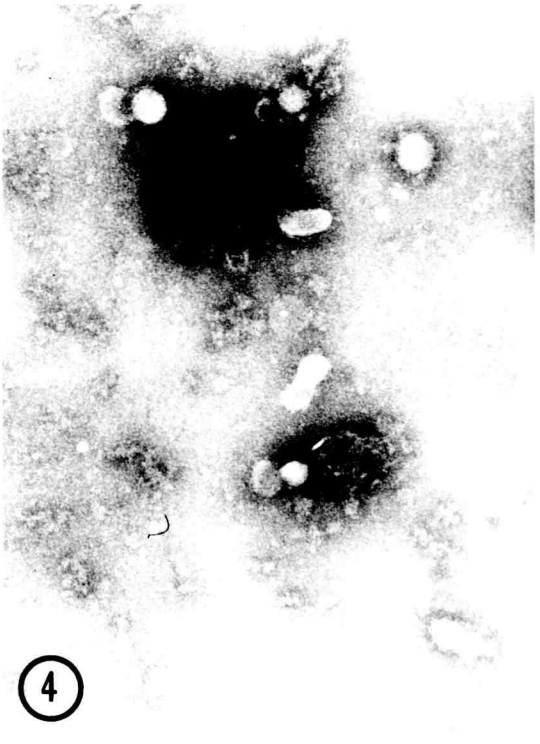

4

shapes and sizes. In glutaraldehyde-fixed virus preparations (see Figures 12, 13, and 14 (1)), the most numerous particle type was bullet shaped (see Figure 15). The approximate size of these particles agreed with the size range (100 to 300 mμ) of OSV previously determined by membrane filtration (Wingfield et al., 1969; Parisot et al., 1965). Two extensively investigated lipid-containing RNA viruses, VSV and rabies virus, have been reported to have sizes and morphology similar to these bullet-shaped particles (Howatson and Whitmore, 1962; Hummeler, Koprowski, and Witkor; 1967). Taking these factors into consideration, the bullet-shaped particle was presumed to be the OSV virion. This presumption was supported in a report by Amend and Chambers (1969). They observed bullet-shaped particles in osmic acid-fixed suspensions of OSV.

In order to describe the size and morphology of these bullet-shaped particles, certain qualifications must be made. The use of glutaraldehyde as a fixative may have caused particle distortion or dissociation of lipoproteins. Sabatini, Bensch, and Barrnett (1963) demonstrated that glutaraldehyde fixation of animal cells preserved fine structure for electron microscope examination. However, treatment of some myxoviruses, i.e. influenza and Newcastle disease viruses, with glutaraldehyde has been reported to cause modifications in the structure of viral envelopes and in the overall appearance of virions (Blough, 1966).

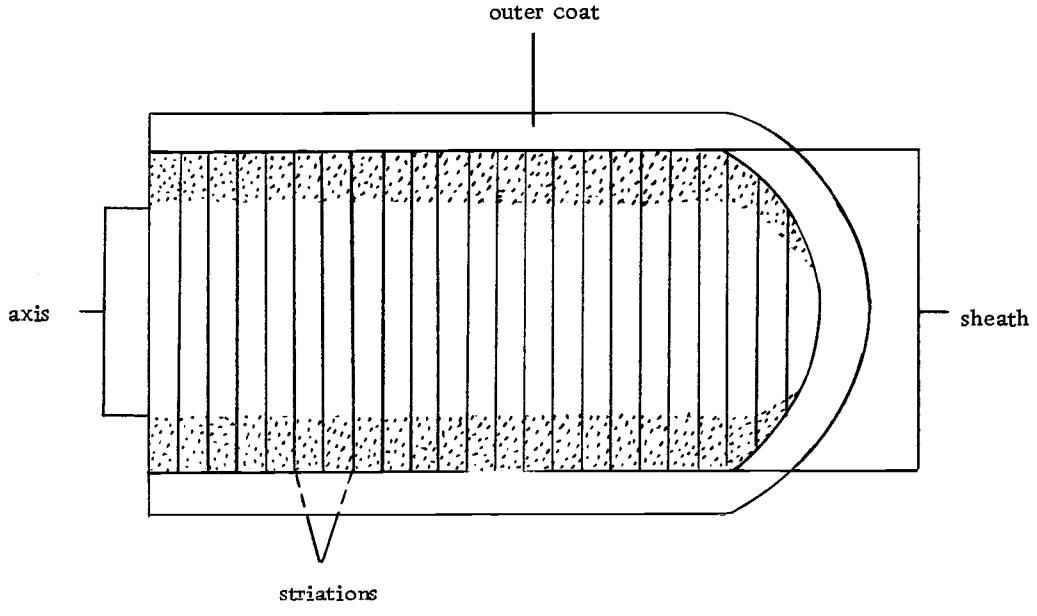

Figure 15. A simplified diagram of the presumed OSV virion demonstrating its shape, morphology, and the terms used to describe its internal structure.

Formaldehyde, another aldehyde fixative, was used by Zwillenberg, Jensen and Zwillenberg (1965) to preserve the structure of the virus of haemorrhagic septicaemia of trout (VHS). These authors identified the structure of this virus as bullet-shaped, and their work has been tentatively accepted by authoritative viral taxonomists (Wilner, 1969). Therefore, the terms which Zwillenberg et al. used to delineate the structures of the VHS virion will be used to describe the bullet-shaped particles observed in suspensions of OSV (see Figure 15).

The dimensions of 37 well defined, intact, bullet-shaped particles were determined. Table 13 contains the distribution of particle sizes. The average length of the virion was 166 mμ, and the average diameter was 98 mμ. Sixty-two percent of these particles had diameters of 95 mμ, with lengths varying from 150 to 175 mμ. Another 24% were 110 mμ in diameter, and had lengths from 130 to 160 mμ.

Bullet-shaped particles sufficiently penetrated by PTA consisted of a striated cylinder (sheath) surrounded on the sides and rounded end by an outer coat. Sheaths were approximately 65 mμ in diameter. Usually a lighter, amorphous region, appearing to contain less PTA, was centrally located along the length of a sheath. This region, known as the axis, was from 40 to 50 mμ in diameter. Sheaths contained between 24 and 25 striations which were 2 to 3 mμ

Table 13. The distribution of particle dimensions among 37 bullet-shaped particles in a glutaraldehyde-fixed suspension of OSV.

Particle Dimensions (mμ)			Number of Particles	Percent of Total Particles Counted
Length		Diameter		
175		95	17	46
150-165		95	6	17
130		110	5	14
140-160		110	4	11
230		90	2	5
163		75	1	2.5
180		100	1	2.5
175		65	1	2.5
Average 166		Average 98	Total 37	
Range 130-230		Range 65-110		

apart. Outer coats surrounding the sheaths were 10 to 14 mμ thick. In some virions, the outer coat appeared to be thicker at the rounded end than on the sides. No clearly defined projections attached to the surface of the outer coat were observed.

Although the morphology of virions in all size groups listed in Table 13 was generally similar to that described above, one significant exception was observed. Two particles with dimensions of 230 x 90 mμ appeared to be rounded at both ends and completely surrounded by an outer coat (see the upper-right corner of Figure 13 and the lower section of Figure 14 (1)).

The presence of truncated virus particles has been reported in negatively stained suspensions of VSV (Hackett, 1964; Huang, Greenawalt, and Wagner, 1966). Truncated or transmissible (T) particles were similar in morphology to bullet-shaped (B) particles, but were one third as long. These authors counted approximately 35 striations in B particles, and between 8 and 14 striations in T particles.

Glutaraldehyde-fixed suspensions of OSV contained a small number of truncated particles which corresponded to the description of the T particles of VSV. They were composed of an outer coat (approximately 12 mμ thick) and an internal component with 8 to 10 striations. Their dimensions were quite variable, ranging from 60 to 90 mμ in length and diameter. (One such particle is located

3/4 inch to the right of the upper PSL sphere in Figure 13, and another can be seen in the lower right corner of Figure 12.)

Very few typical bullet-shaped particles were observed in unfixed suspensions of OSV (see Figure 14 (2), (3) and (4)). In these preparations, the most numerous type of particle was composed of two closely associated, roughly spherical structures. Each spherical structure was approximately 80 mμ in diameter, and together they formed a particle about 160 mμ in length and 80 mμ in diameter. Four of these particles are in Figure 14 (4).

Two particles which most resembled the bullet-shaped particles observed in glutaraldehyde-fixed preparations are located in the middle-right part of Figure 14 (2) and at the top of Figure 14 (3). They do not contain visible internal structures, but have dimensions of approximately 180 x 95 mμ. Both particles have faintly visible projections, 10 mμ in length, extending from their surface.

Another type of bullet-shaped particle was observed that appeared to contain two or more major subunits. This particle (see lower part of Figure 14 (3)) had dimensions of 230 x 130 mμ. It was composed of a "U"-shaped base (150 x 130 mμ) attached to two lobular structures which appeared to surround an irregularly shaped sphere (80 mμ in diameter). A "U"-shaped particle resembling the base of the multi-structured particle is located in the center of Figure 14 (2).

Due to the apparent disagreement between the appearance of fixed and unfixed suspensions of OSV, the presumptive identification of the OSV virion as a bullet-shaped particle is made more tenuous. A likely explanation of this discrepancy, however, is suggested by the extreme sensitivity of OSV to osmotic changes (see Experimental Results, Virus Purification). Surface tension effects caused by drying virus suspensions on Formvar-coated grids could have disrupted or distorted unfixed virus particles.

Conclusive electron microscopic identification of the OSV virion must wait until quantitative particle counts are performed on suspensions of OSV and correlated with the viral infectivity of these suspensions.

DISCUSSION

Research described in this thesis and in a report by Wingfield et al. (1969) has characterized the OSV as a lipid-containing, RNA virus. Also, a bullet-shaped particle with average dimensions of 166 x 98 mμ was presumptively identified as the OSV virion. Similarities between OSV and other RNA-, lipid-containing animal viruses (especially the bullet-shaped viruses, VSV and rabies) will be noted in the following discussion to support or explain some of the properties elucidated for the OSV.

The data reported in the EXPERIMENTAL RESULTS section were divided into five sections, including Virus Purification, Determination of OSV Density, Characterization of OSV Nucleic Acid, Cross Plaque Neutralization Tests, and Electron Microscopy. The following discussion of experimental results will also be divided into these categories. However, in some cases, results previously described in separate sections will be discussed together in order to explain a particular experimental observation.

Procedures used to purify virus contained in growth medium from OSV-infected cell cultures were differential centrifugation alone, or in combination with nuclease treatment and rate-zonal sucrose gradient centrifugation. Three properties of OSV suspensions greatly affected the recovery of virus infectivity and the degree of virus

purity resulting from these purification procedures. Virus in cell culture fluid had a pronounced tendency for aggregation, or complex formation, with non-viral material during differential centrifugation. Differentially centrifuged virus suspensions formed four visible bands when subjected to rate-zonal or isopycnic sucrose gradient centrifugation. Considerable virus infectivity was lost when OSV was banded in sucrose gradients, and when the sucrose concentration in the gradient fractions was reduced by dilution and/or dialysis.

The tendency for viral aggregation or complex formation was most pronounced when differential centrifugation consisted of a low-speed cycle of 2,200 x g for 30 min and a high-speed cycle of 55,000 x g for 60 min. No statistically significant loss of virus infectivity was detected during these two steps. However, when the resuspended virus pellet was subjected to a centrifugal force of only 130 x g for 10 min, approximately 80% of the infectivity was sedimented.

Experimental evidence indicated that the formation of easily sedimented virus complexes was caused by pelleting OSV in the presence of host cell debris during the high-speed cycle of differential centrifugation. A modified differential centrifugation technique, utilizing sedimentation cushions of 95% glycerol and rate-zonal discontinuous glycerol gradient centrifugation, was employed (see Table 2). This method delayed virus pelleting until low density cellular material and proteins were removed from the virus suspension. It

yielded a suspension unaffected by centrifugation at 130 x g for 10 min and increased the percent recovery of virus infectivity by approximately 50%.

In an article describing a purification procedure for rabies virus, Sokol et al. (1968) reported that rabies virus formed strong complexes with host cell debris. Sokol and co-workers were able to overcome this difficulty by collecting infectious growth medium from infected cell cultures showing no cellular degeneration, and by selectively precipitating out the virus from this medium with zinc acetate. Nevertheless, when 10% heat-inactivated calf serum was used in the growth medium, about 25% of the serum proteins coprecipitated with the virus during zinc acetate treatment. The resulting virus suspensions behaved almost exactly like differentially centrifuged suspensions of OSV; pelleting the rabies virus by high-speed centrifugation converted about 80% of the virus to a form which could be sedimented by low-speed centrifugation.

Sokol and co-workers attempted to improve the recovery of dispersed virus by treating virus pellets with sonication and homogenization. Neither technique was able to appreciably disperse virus aggregates. Also, cushions of 60% sucrose were employed during high-speed centrifugation to reduce the effects of virus pelleting; however, the sensitivity of rabies virus to osmotic pressure changes lessened the effectiveness of this method.

Believing that the presence of serum proteins during the virus-pelleting step contributed to viral aggregation, Sokol et al. substituted 0.4% BSA for 10% calf serum in the growth medium. This substitution enabled them to precipitate rabies virus from infectious medium with only 2 to 5% protein coprecipitation, and to essentially eliminate viral aggregation.

Although 5% agamma calf serum was replaced by 0.4% BSA in the complete MEM used for OSV propagation, no noticeable reduction in the amount of virus aggregation during differential centrifugation was observed. This result was not surprising, since both BSA and low molecular weight cell debris were present during pelleting of the OSV. BSA (0.4%) was substituted for serum in the complete MEM used to propagate OSV for all experiments, except virus density determinations. BSA did not reduce the yield of infectivity during virus propagation, and the composition of a 0.4% solution of BSA was considered to be less complex and more uniform than that of commercial calf serum.

Differentially centrifuged suspensions of OSV were fractionated into four visible bands in sucrose gradients by either isopycnic or rate-zonal centrifugation. The gradients contained a top band, two closely associated middle bands, and a diffuse bottom band. The locations of each set of visible bands corresponded to the positions of three infectivity and/or ^{32}P-radioactivity peaks detected in sucrose gradients. In virus density determinations, the top, middle, and bottom

infectivity peaks contained approximately 1, 85 and 6% of the total detected infectivity, respectively. Sucrose densities determined for the top, middle and bottom infectivity peaks were 1.10, 1.16, and 1.20 g/cm^3 (see Figure 1). Rate-zonal centrifugation of ^{32}P-labeled OSV produced peaks of infectivity and radioactivity in regions of the gradient with sucrose densities of about 1.05, 1.14, and 1.20 g/cm^3.

Positive identification of the four visible bands or corresponding infectivity and radioactivity peaks cannot be made at this time. Virus- and host-cell-specific complement-fixing antiserum and techniques for electron microscope examination of OSV suspended in concentrated sucrose solutions must be developed before the composition of these fractions can be established. However, evidence obtained in this research and the reported properties of other lipid-containing, RNA viruses will be used to presumptively identify the top, middle, and bottom bands observed in sucrose gradients.

Since the major infectivity peak in isopycnic sucrose gradients was associated with the two middle visible bands, it was apparent that one or both of these bands represented the virus band. The identity of the virus band was indicated by rate-zonal centrifugation of ^{32}P-labeled OSV. Peaks of infectivity and radioactivity were detected at a sucrose density of about 1.14 g/cm^3 (the location of the two middle bands). However, infectivity was associated with the faster sedimenting region of the peak of radioactivity; the maximum values of the two

peaks were separated by one 1.0-ml fraction. The two visible bands were approximately 4 mm or 1.5 ml apart in the gradient. Thus, it is likely that the faster sedimenting of the two middle bands was infectious, and the slower band was non-infectious. For convenience, both middle bands will be referred to collectively as the "virus band" in the remainder of this discussion.

Other lipid-containing, RNA viruses have been reported to form heterogeneous virus bands when fractionated in sucrose gradients. The behavior of rabies virus, described by Sokol et al. (1968), most closely parallels the results observed with ^{32}P-labeled OSV. Partially purified suspensions of ^{3}H-uridine-labeled rabies virus, harvested from cell cultures showing no visible CPE, formed a homogeneous band when subjected to rate-zonal sucrose gradient centrifugation. Virus labeled and purified in the same manner, but harvested after extensive host-cell degeneration had occurred, formed a heterogeneous band. The maximum value of the infectivity peak, in terms of pfu per ml, was about one 1.0-ml fraction lower in the gradient than the maximum cpm of the radioactivity peak. Similar distributions of radioactivity and infectivity in sucrose gradients were observed for ^{32}P- or ^{3}H-uridine labeled Rous sarcoma virus (RSV, Bryant strain) (Robinson, Pitkanen, and Rubin; 1965).

A possible explanation for the heterogeneity of virus bands obtained by rate-zonal sucrose gradient centrifugation of rabies virus

and OSV is suggested by extensive research performed on the non-infective interfering component (T particles) of VSV. Suspensions of VSV, containing a high percentage of T particles, are usually produced by infecting chick embryo cell cultures with high pfu/cell ratios (1.5 to 100) and performing four or five undiluted serial virus passages (Huang, Greenawalt, Wagner; 1966; Hackett, 1964).

When suspensions of ^{32}P- or ^{3}H-uridine-labeled VSV containing T particles were subjected to rate-zonal sucrose gradient centrifugation, two closely associated radioactivity peaks were detected. The infectious, faster sedimenting peak contained less radioactivity than the slower sedimenting, noninfectious peak (Brown et al., 1967; Huang et al., 1966). Electron micrographs of suspensions obtained from the slower peak show a high concentration of truncated particles. These short particles have dimensions of approximately 65 x 65 mμ, compared to 65 x 180 mμ for the bullet-shaped particles (B particles) obtained from the infective peak (Huang et al., 1966).

Truncated particles have been observed in electron micrographs of rabies virus (Sokol et al., 1968) and OSV suspensions which formed heterogeneous virus bands when fractionated by sucrose gradient centrifugation. In suspensions from a heterogeneous rabies virus band, Sokol and co-workers counted approximately 10% more short particles (around 75 mμ in length as compared with the average particle length of 180 mμ) than were seen in virus suspensions obtained

from homogeneous virus bands. Approximately 10% of the particles observed in electron micrographs of differentially centrifuged and glutaraldehyde-fixed suspensions of OSV were truncated, i.e., about 65 mμ in length (see Figure 12).

Although VSV, rabies virus, and OSV may be bullet-shaped viruses with truncated particles associated with their heterogeneous virus bands, several properties of OSV and rabies virus indicate that the slow sedimenting component of their virus bands is not the same as that reported for VSV. The sedimentation coefficients of the infective and noninfective components of rabies virus and OSV appear to be more similar than those of the components of VSV. As was stated earlier, the maxima of the infectivity and radioactivity peaks observed for ^{3}H-uridine-labeled rabies virus (Sokol et al., 1968) and ^{32}P-labeled OSV were only about one 1.0-ml fraction apart after rate-zonal sucrose gradient centrifugation. Neurath, Wiktor, and Koprowski (1966) reported the sedimentation coefficient of rabies virus as 600 S. Bradish, Brooksby and Dillon (1956) determined the sedimentation coefficients of the infective and noninfective components of VSV to be 625 S and 330 S, respectively.

Virus-band heterogeneity reported for rabies virus by Sokol et al. (1968) was possibly a function of host cell competence. Heterogeneous suspensions of rabies virus were obtained from cell cultures infected with the same pfu/cell ratio and for the same period as

cultures yielding homogeneous virus suspensions. However, older cell cultures showing cell degeneration were used to propagate heterogeneous virus suspensions.

Host cell degeneration during OSV propagation may have been associated with potentially high pfu/cell ratios. OSV was routinely propagated by infecting cell cultures with a pfu/cell ratio of between 0.003 and 0.005. This multiplicity of infection was much lower than that used to induce T-particle production with VSV. However, the infection process for OSV was allowed to proceed for 72 to 96 hours until extensive CPE was observed. During this period, cells not initially infected by OSV could have been infected by progeny virus at potentially high pfu/cell ratios. (Wingfield et al. (1969) reported that progeny OSV were detected in cell culture fluid at 24 to 48 hours PI.)

Even though conditions under which heterogeneous OSV suspensions were produced may have been similar with respect to the multiplicity of infection required for T-particle production in VSV propagation, the slower sedimenting component of the OSV virus band probably had different physical properties than the interfering component of VSV. Wagner, Schnaitman and Snyder (1969) found that the B and T particles of VSV had the same density in cesium chloride gradients. Assuming that the two visible bands observed in sucrose gradients after isopycnic centrifugation of OSV suspensions represented the infective and noninfective components, then these

components have different densities. This proposed difference in density between the two components of the OSV virus band indicates that the less dense, noninfectious component may consist of incomplete virus particles which do not have the same proportionate amounts of nucleoproteins, proteins and lipoproteins as do infective particles.

The diffuse bottom band observed in sucrose gradients subjected to isopycnic centrifugation was located at a density of 1.20 g/cm^3 and was assocated with about 6% of the virus infectivity detected in the gradients. Since this band usually contained large amounts of clumped material and relatively little infectivity, it was assumed to be composed of host cell debris and virus aggregates formed during differential centrifugation. Support for this assumption was provided by experiments in which complex formation was reduced by differential centrifugation employing glycerol pads and a discontinuous glycerol gradient (see Table 2). When resulting virus suspensions were subjected to rate-zonal sucrose gradient centrifugation, no visible bottom band was observed.

A larger amount of virus infectivity was associated with the bottom bands after rate-zonal sucrose gradient centrifugation of ^{32}P-labeled OSV (see Figure 3). Bottom bands migrated to a sucrose density of about 1.20 g/cm^3, the density of this band after isopycnic centrifugation. The percentages of infectivity detected in the bottom and middle (virus) bands of the two gradients shown in Figure 3 were

26 and 69%, and 42 and 55%, respectively.

An increase in the amount of virus infectivity found in the faster sedimenting bottom band could have been caused by the cell line used for virus propagation or radiation effects from ^{32}phosphorous. In virus density determinations, where only six percent of the infectivity was in the bottom band, OSV was propagated in cell line SSE-30 (derived from sockeye salmon embryonic tissue, the species from which OSV was isolated). The chinook salmon embryo cell line, CHSE-214, was used to propagate ^{32}P-labeled OSV because this line yielded infectious culture medium with higher virus concentrations. It is possible that virus particles produced in the chinook salmon cell line had a greater tendency to complex with cell debris or to remain bound to cell membranes. Another factor, which could have caused the same effects, was possible cell damage from the beta particles of ^{32}phosphorous.

Sokol et al. (1968) and Neurath et al. (1966) detected a small fraction of rabies virus infectivity which sedimented faster than the major peak of infectivity in sucrose gradients centrifuged by the rate-zonal method. Both groups of researchers observed this effect only when rabies virus was propagated in the presence of calf serum, precipitated from infectious culture fluid with zinc acetate, and was pelleted during high-speed centrifugation. Neurath et al. eliminated this lower fraction by subjecting unconcentrated rabies virus suspensions

to rate-zonal centrifugation. As stated above, in connection with OSV aggregation, Sokol et al. were able to substantially reduce rabies virus aggregation by propagating virus in the presence of 0.4% BSA rather than calf serum. When partially purified rabies virus, produced in the presence of BSA was fractionated in a sucrose gradient, a single peak of virus infectivity was obtained. Thus, by eliminating conditions conducive to rabies virus aggregation, both Neurath et al. and Sokol et al. were able to remove the faster sedimenting virus fraction.

In addition, Sokol et al. (1968) found that rate-zonal sucrose gradient centrifugation of cell-associated rabies virus suspensions yielded a visible, noninfective, band which sedimented faster than the virus band. Cell-associated rabies virus was obtained by freezing and thawing washed infected cell suspensions. They considered this bottom band to be composed of cellular debris because suspensions derived from uninfected cells, treated in the same manner, formed a visible band in the same region of the sucrose gradient.

Since infectious culture medium used for OSV purification contained large amounts of host cell debris, these latter observations of Sokol et al. give strong support to the contention that the bottom band seen in fractionated suspensions of OSV is of cellular origin. In an effort to gain additional evidence, a suspension of uninfected cells from cell line CHSE-214 was subjected to three cycles of freezing and

thawing (-20° to 20°C). The resulting suspension was partially purified and fractionated in a sucrose gradient in the same manner used for OSV purification. No virus or bottom bands were observed in the sucrose gradient. Possibly the freeze-thaw method used to disrupt the fish cells was not effective in reproducing the degree of cellular degeneration obtained during OSV propagation.

Bands which sedimented faster than the majority of virus infectivity during rate-zonal sucrose gradient centrifugation have also been reported for VSV (Huang et al., 1966) and influenza virus (strain BEL: Barry and Davies, 1968). Both sets of authors assumed that these bands were composed of viral material. Huang et al. based their assumption on a report by McCombs, Benyesh-Melnick and Brunschwig (1966). When suspensions of VSV were banded in cesium chloride gradients by isopycnic centrifugation, McCombs et al. observed three infective bands. The most dense band, with the least infectivity, contained disrupted virus particles as determined by electron microscope examination. Huang et al. considered this most dense band to be analogous to the noninfectious, faster sedimenting band they obtained by fractionating VSV suspensions in sucrose gradients. Barry and Davies (1968) extracted RNA from influenza virus associated with a virus band and a faster sedimenting, infectious band in sucrose gradients. The sedimentation coefficients of RNA molecules from both bands were quite similar. These authors

theorized that the lower band was composed of virus particles containing large amounts of envelope material.

In summary, it is not unusual to find an infectious or non-infectious component which sediments faster than the virus band during rate-zonal sucrose gradient centrifugation of RNA-, lipid-containing viruses. Although explanations for these components vary, the interpretations presented by Neurath et al. (1966) and Sokol et al. (1968) for rabies virus seem to correspond most closely with observations made with OSV. Thus, the bottom band seen in sucrose gradients containing OSV suspensions fractionated by isopycnic or rate-zonal centrifugation appears to be composed of a mixture of host cell debris and aggregated virus.

In addition to the bottom band and the two middle bands, a fourth visible band was observed at the top of sucrose gradients containing fractionated OSV suspensions. After isopycnic centrifugation in virus density determinations, this top band was in the same relative position as an infectivity peak containing approximately 1.0% of the total virus infectivity (see Figure 1). Rate-zonal sucrose gradient centrifugation of ^{32}P-labeled OSV suspensions also produced a top band which was associated with a small amount of radioactivity (see Figure 3).

The apparent propensity of OSV for forming aggregations with cellular debris or serum proteins probably accounts for the infectivity associated with the top band. Presence of ^{32}P-labeled virus material

or host-cell lipoproteins and/or RNA (Sokol et al., 1968) could explain the radioactivity detected in this band.

Density determinations of OSV were performed by isopycnic sucrose gradient centrifugation. As mentioned above, approximately 85% of the virus infectivity in the gradients was at a sucrose density of 1.16 g/cm^3. This density value agrees with the densities reported for other RNA-, lipid-containing viruses in sucrose gradients, i.e., VSV (McCombs et al., 1966), rubella virus (McCombs and Rawls, 1968), and RSV (Robinson et al., 1965).

Most lipid-containing RNA animal viruses have been reported to have densities in sucrose gradients between 1.15 and 1.18 g/cm^3. It is difficult to know whether this range of densities can be attributed to differences in the physical properties of these viruses or to the techniques used to determine virus densities. One very important factor which must be taken into consideration when comparing the densities of various viruses is the solvent used in sucrose gradients. McCombs and Rawls (1968) found that density values determined for rubella virus were greater when sucrose gradients were prepared in Tris-EDTA buffer (virus density, 1.18 g/cm^3) than when similar gradients were dissolved in water (virus density, 1.16 g/cm^3). Another example of this "solvent effect" is apparent in sucrose density values obtained for RSV by two different groups of researchers. Kahler et al. (1954) banded RSV in sucrose gradients dissolved in

water and reported a virus density of 1.15 g/cm^3. Robinson et al. (1965), using similar techniques with sucrose gradients prepared in Tris-EDTA buffer, found the density of RSV to be 1.16 g/cm^3.

Throughout this discussion, the properties of OSV have been compared with similar properties reported for rabies virus. The densities of OSV and rabies virus in sucrose gradients are 1.16 and 1.17 g/cm^3 (Sokol et al., 1968), respectively. Although the difference between these two densities is probably not significant, it is of interest that Sokol et al. used Tris-EDTA buffer in their sucrose gradients and OSV densities were determined in gradients dissolved in water.

Only 16 to 28% of the OSV infectivity present in suspensions subjected to rate-zonal sucrose gradient centrifugation was detected in the resulting virus bands (see Tables 2 and 3). Distribution of aggregated virus to positions in the gradient other than the virus band could have accounted for part of this loss of infectivity. In addition, OSV may have been inactivated by high sucrose concentrations, or by changes in osmotic pressure resulting from reductions in sucrose concentrations. The amount of sucrose in samples obtained from gradients was rapidly decreased during the serial dilution step of infectivity assays.

Virus in fractions collected from sucrose gradients was also exposed to osmotic pressure changes when sucrose was removed by dialysis and/or repeated sedimentation by high-speed centrifugation.

Between 85 and 90% of the infectivity assayed in gradient fractions was lost during the sucrose removal step (see Table 4).

Sokol et al. (1968) found that rabies virus was similarly sensitive to osmotic changes once virus suspensions were fractionated in sucrose gradients. The infectivity of VSV has also been reported to be drastically reduced by exposure to high sucrose concentrations. Cooper and Bellett (1959) exposed suspensions of VSV to various media for 21 hours at 0°C. Suspensions in Earle's saline contained 49% of the initial infectivity; however, in the presence of 0.88 M (30%) sucrose, less than 0.1% of the virus infectivity survived. After fractionating suspensions of VSV by rate-zonal sucrose gradient centrifugation, Huang et al. (1966) were able to recover only 10% or less of the infectivity originally layered on the gradients.

The discussion of experimental results, which characterized the nucleic acid extracted from purified suspensions of OSV, will be divided into three parts. In the first part, the spectrophotometric characteristics of viral nucleic acid solutions will be discussed. Next, an interpretation of the experimental evidence employed to identify the type of viral nucleic acid will be presented. Some of this evidence will be used in an effort to eliminate the possibility that viral nucleic acid solutions were contaminated by cellular DNA or RNA. The third part will involve analyses of data obtained in viral RNA sedimentation coefficient and base composition determinations.

UV spectra of solutions containing viral nucleic acid extracted by the PCA or phenol methods were surprisingly similar (Figures 2a and 5). Absorbance at the lower wavelengths (230-250 mμ) was quite high for nucleic acid solutions, indicating that relatively large amounts of protein, i.e. peptide bands, were present (McLaren and Shugar, 1964). In addition, the low 260/280 mμ absorbance ratios calculated for these solutions, ranging from 1.13 to 1.37, were further evidence of protein contamination (Chaykin, 1966). Since no effort was made to remove dissolved proteins and amino acids from PCA extracts, this result was not unexpected. However, the phenol method employed to extract viral nucleic acid has been reported to remove most viral proteins (Gierer and Schramm, 1956).

The nucleoprotein complex associated with OSV particles was apparently resistant to complete dissociation during extraction with buffer-saturated phenol at 4°C. Nucleic acid solutions from purified suspensions of avian myeloblastosis virus (AMV, an RNA-, lipid-containing virus) were extracted by the same phenol method, in the same laboratory, and with the same chemicals. The AMV nucleic acid solutions had the following absorption characteristics at UV wavelengths: 260/280 mμ ratios of around 1.98, maximum absorbance at 260 mμ, and minimum absorbance at 230 mμ (Deeney, 1969).

In spectrophotometric analyses of PCA extracts from unlabeled OSV suspensions, the presence of proteins probably affected the

accuracy of total nucleic acid determinations. Total nucleic acid concentrations were estimated by measuring absorbance at 260 mμ. Interference by peptide bands at wavelengths from 230 to 240 mμ and by amino acids, tyrosine and tryptophan, in the UV region between 260 and 280 mμ could have resulted in erroneously high readings at 260 mμ.

Another experimental observation which may have been related to protein contamination was the colorimetric detection of RNA and DNA in PCA extracts of unlabled OSV nucleic acid. Although the concentrations of RNA were at least 14 times higher than that of DNA, the presence of both types of nucleic acid indicated contamination by cellular DNA. However, Hutchison and Munro (1961) reported that the diphenylamine test reacts with proteins present in DNA solutions, producing colors which cause incorrectly high absorbance values.

Nucleic acid extracted from ^{32}P-labeled OSV, purified in the same manner as unlabeled virus, was banded in cesium sulfate gradients (see Figure 7). No significant peaks of radioactivity or absorbance (at 260 mμ) were detected in the region of these gradients corresponding to cesium sulfate densities between 1.40 and 1.50 g/cm^3. Most double-stranded cellular or viral DNA has been found to band at densities of 1.42 to 1.45 g/cm^3 (Szybalski, 1968).

Also, ^{32}P-labeled OSV nucleic acid was subjected to rate-zonal glycerol gradient centrifugation (see Figure 8). One half the volume of

each fraction, collected from this gradient, was treated with RNase (17 μg/ml) at 30°C for 40 min. The TCA-precipitable ^{32}P-radioactivity detected in the RNase-treated portions of the fractions was 85 to 97% less than that measured in the untreated portions. This experiment indicated that very little or no DNA was present in the OSV nucleic acid solution and that the viral nucleic acid was single-stranded RNA.

Although the aforementioned data provides reasonable assurance that the procedures employed in virus purification were effective in eliminating host-cell DNA, it offers no indication that cellular RNA was also eliminated. However, indirect evidence that extracted viral nucleic acid was relatively free of cellular RNA was provided by isopycnic cesium sulfate centrifugation of phenol extracted, ^{32}P-labeled OSV nucleic acid (see Figure 7). In the two gradients represented in Figure 7, major peaks of 1.58 and 1.59 g/cm^3, respectively. In addition, approximately 90% of the radioactivity in the two gradients was below a density of 1.62 g/cm^3. Reported densities for the RNA of other RNA viruses include 1.66 g/cm^3 for rabies virus (Sokol et al., 1969), 1.65 g/cm^3 for polio virus (Bishop, Summers, and Levintow; 1965), and 1.68 g/cm^3 for NDV (Kingsbury, 1966). Therefore, cesium sulfate densities obtained for OSV nucleic acid were too high to represent DNA, but were lower than commonly observed with viral RNA.

The most obvious explanation for the low density of OSV RNA was indicated by the low 260/280 mμ absorbance ratios of viral RNA solutions before and after banding in cesium sulfate gradients. As mentioned above, low 260/280 mμ ratios indicate the presence of relatively large amounts of RNA-associated proteins. Thus, proteins complexed with the OSV RNA probably decreased the density of these molecules.

OSV nucleoprotein complexes were apparently resistant to dissociation by phenol extraction at 4^{o}C. Cellular RNA extracted from ^{3}H-labeled chick myeloblasts with the same phenol extraction technique was banded at a cesium sulfate density of 1.65 g/cm^{3} (see Figure 6). Although RNA from the host cell line, CHSE-214, was not subjected to cesium sulfate centrifugation, it is reasonable to expect that this RNA would have banded at a density of around 1.65 g/cm^{3}. Since about 90% of the radioactivity detected in gradients containing ^{32}P-labeled OSV RNA was at densities of 1.62 g/cm^{3} or lower, it is likely that most of the radioactivity represented viral nucleoprotein complexes and not cellular RNA.

The validity of the experimental data which established the identity of OSV nucleic acid as RNA would not have been affected by the presence of host-cell RNA in viral nucleic acid solutions. However, determinations of the sedimentation coefficient and base composition of OSV RNA could have been influenced by such contamination.

Rate-zonal centrifugation of ^{32}P-labeled OSV RNA in a 10 to 30% glycerol gradient was used to determine the sedimentation coefficient of the viral RNA. Chick myeloblast nucleic acid, labeled with ^{14}carbon, was used as a marker. ^{32}P-radioactivity was distributed in a diffuse peak with the maximum radioactivity value at 26 S and a definite shoulder at 37 S. Due to the heterogeneous nature of the viral RNA, no definite conclusions as to the sedimentation coefficient of OSV RNA can be made.

Several factors could have contributed to the apparent heterogenicity of the viral RNA. Protein complexed with RNA molecules could have caused variations in the sedimentation rates of these complexes. Ribonuclease treatment during OSV purification may have nicked the viral RNA in nucleocapsids. Although RNase and DNase treatment (30 μg/ml each, for 60 min at 25°C) of OSV suspensions caused no reduction in virus infectivity, Sokol et al. (1969) found that RNase treatment (100 μg/ml for 30 min at 33°C) of rabies virus nucleocapsides caused extracted RNA to have a lower sedimentation coefficient and more heterogenicity than RNA from non-treated nucleocapsides. Another factor which may have induced the breakdown of OSV RNA molecules was osmotic disruption of virus particles when fractions from sucrose gradients were diluted during phenol extraction. Possible contamination of OSV RNA solutions with cellular RNA could also have contributed to the heterogeneous appearance of the viral RNA.

The pronounced shoulder of ^{32}P-radioactivity located at the 37 S region of the glycerol gradient may represent a fraction of the OSV RNA which was intact and not associated with proteins and non-viral RNA. If this is the case, then a sedimentation coefficient value of 37 S for OSV RNA approximates similar values determined for single-stranded RNA extracted from the infective particles of VSV and rabies virus. Brown et al. (1967) extracted RNA from the infective and non-infective components of VSV banded in sucrose gradients. RNA from infective VSV particles was found to be 38 S, and the noninfective particles yielded 18 to 20 S RNA molecules. The sedimentation coefficient of rabies virus RNA was determined to be 45 S by Sokol et al. (1969).

Nucleotide composition analyses were performed on ^{32}P-labeled OSV RNA banded in cesium sulfate gradients. The single fraction containing most of the ^{32}P-radioactivity from the gradient presented in Figure 7b was used for these analyses. This fraction was located at a cesium sulfate density of 1.59 g/cm^3, which was, as stated earlier, a much lower density than would be expected for the banding of cellular RNA. Nevertheless, the validity of the base composition values determined for OSV RNA could have been strengthened if the RNA used in these analyses had been obtained from glycerol gradients containing a homogeneous band of viral RNA.

Average base composition values, determined from the results

of three analyses, were C, 25.8 ± 0.6%; A, 23.0 ± 0.6%; U, 27.7 ± 0.6%; and G, 23.4 ± 0.4%. The average A + U/G + C ratio was 1.03. These data suggest that the RNA of OSV is single-stranded.

Most reported base compositions of myxoviruses and paramyxoviruses also show percentages of U to be significantly higher in relation to the other three bases. For example, Cocito, Ladurou, and Somer (1963) reported the base composition for influenza virus (PR8) RNA as C, 22.8%; A, 26.1%; U, 29.6%; and G, 21.5%. NDV RNA was reported to have a base composition of C, 23.2%; A, 20.1%; U, 31.2%; and G, 25.4% by Kingsbury (1966).

Base composition analyses performed on RNA from infectious VSV particles by Brown et al. (1966) indicated that complementarity was present in VSV RNA. The composition of A (29.3%) was almost equal to that of U (28.7%), and the percentage of G (20.9%) was almost the same as C (21.1%). However, this apparent complementarity was considered to be fortuitous because of the sensitivity to RNase and the sedimentation coefficient of VSV RNA.

In recent years many attempts have been made in various laboratories to develop rabbit immune serum to OSV, SRCD virus, and IHN virus. As of this writing, no published articles describing the results of quantitative serological techniques with such antisera have appeared in the literature. Parisot et al. (1965) stated that SRCD virus was inactivated by rabbit and fish anti-SRCD serum; however,

these authors presented no data to support their statement.

A likely explanation for this apparent lack of success is the low concentrations of viral antigens which have been used for rabbit injections. The usual practice has been to repeatedly inject rabbits with unconcentrated infectious cell culture fluid emulsified with adjuvant (Amend, 1969). In the experiments reported in this thesis, rabbit antisera were produced by injecting rabbits with differentially centrifuged suspensions of virus containing between 5.0×10^8 and 2.0×10^9 $TCID_{50}$/ml. Two series of injections about 14 days apart were performed. The 50% plaque neutralization end points determined for antisera against each of the three viruses ranged from 1/140 to 1/3900, and were proportional to the total infectivity of the respective virus suspensions injected into the rabbits.

The importance of antigen concentration for producing immune serum in rabbits is well documented. Farr and Dixon (1960) investigated the effects of varying antigen concentrations in ^{131}I-labeled BSA solutions, with and without adjuvant, on rabbit antibody production. They found that small volumes with high concentrations of antigen were more than twice as effective for initiating detectable antibody production than 10- to 20-fold higher volumes with the same total amount of antigen. In addition, IV injections of antigen solutions without adjuvant required almost 100 times more antigen to produce the same degree of immune response as solutions containing adjuvant

injected subcutaneously.

Antigenic relationships between OSV, IHN virus, and SRCD virus were examined using cross plaque neutralization tests; each of the three antisera was tested versus the three viruses. The 50% plaque neutralization end points determined for OSV and IHN virus were not significantly different in all three sets of tests (two tests per antiserum). Therefore, these two viruses, which were both isolated from sockeye salmon, appear to be antigenically very closely related.

Also, results of the cross plaque neutralization tests suggest that the SRCD virus has some antigenic properties in common with the other two viruses. The 50% plaque neutralization end points for SRCD virus were from 1.6 to 2.5 times higher than those obtained with OSV and IHN virus in all tests performed, even when the SRCD virus was not the homologous one.

SRCD virus was originally isolated from chinook salmon and the cell line, CHSE-214, used to plaque viruses in the neutralization tests was derived from chinook salmon embryos. Thus, the consistently high end points determined for SRCD virus may have been related in some way to virus-host cell specificity, rather than to antigenic differences.

The effect of host cells on serum neutralization of VSV was investigated by Kjellén and Schlesinger (1959). VSV was treated with increasingly higher dilutions of antiserum, and surviving virus was

assayed on both chick embryo fibroblasts (CE) and human leukemic bone marrow (MCN) cell cultures. Virus concentrations detected with CE cells were 10- to 1000-fold higher than detected with MCN cells. Although further experiments by Kjellén and Schlesinger suggested that these observations were associated with virus-antibody interactions alone, they were unable to offer any definitive explanations.

However, Lafferty (1963) theorized that the above observations were due to "nonneutralizing" antibodies present in the antiserum used to neutralize VSV. He characterized these antibodies as capable of combining with virus particles, but unable to prevent virus adsorption and penetration. "Nonneutralizing" antibodies would then compete with neutralizing antibodies for antigenic sites on a virion. Since neutralizing antibody-virus complexes are known to adsorb to host cells, Lafferty suggested that the host cell plays an important role in recognizing whether an antibody-virus complex is neutral.

In the case of cross-plaque neutralization tests with the salmonid viruses, only one cell line was used as host cell. Nevertheless, the importance of host cell recognition of virus-antibody complexes, proposed by Lafferty, may explain higher plaque neutralization end points determined for SRCD virus. Adsorption to and/or penetration of the cells of cell line CHSE-214 may be more specific for SRCD virus than for the other two viruses. Thus, the same type of antibody which combines with an SRCD virus particle and renders it neutral may

complex with an OSV or IHN virus particle and still leave it infectious.

Another factor influencing the proportionately higher degree of SRCD virus neutralization could have been related to the number of virus particles required to produce one pfu in cell line CHSE-214. Each of the dilutions of normal and immune sera employed in a cross plaque neutralization test was combined with sufficient OSV, IHN virus, or SRCD virus to give approximately 100 plaques per bottle in the normal serum controls. Because the host cells were derived from the natural host for SRCD virus, it is possible that fewer SRCD virus particles were required to produce the same number of plaques as OSV and IHN virus particles. If concentrations of virus particles were lower in tests performed with SRCD virus than in tests with the other two viruses, then proportionately less neutralizing antibody, i.e., higher antiserum dilutions, would reduce the number of SRCD virus pfu by 50%.

An additional explanation for the results of these serological tests was elucidated by Chubb and Biggs (1968). They reported that certain strains of RSV were neutralized by homologous antiserum to a lesser degree than were other, heterologous strains. Experimental evidence suggested that variations in the amounts of virus components and phenotypic mixing in the homologous virus populations were responsible for their observations. An isolate from one strain of RSV was cloned 10 times and antiserum was produced against the final

clone. This antiserum was able to substantially neutralize the cloned virus, but caused only limited neutralization of the parent strain. Therefore, suspensions of OSV and IHN virus used in plaque neutralization tests may have contained more incomplete, noninfectious virus particles than suspensions of SRCD virus.

The close antigenic relationship between OSV, IHN virus, and SRCD virus, indicated in cross-plaque neutralization tests, is in agreement with reported morphological similarities of these three viruses. Amend and Chambers (1969) examined osmic acid-fixed, negatively stained suspensions of IHN virus, SRCD virus, and OSV with an electron microscope. In suspensions of all three viruses, they observed bullet- or oval-shaped virus-like particles. The average dimensions of between 22 and 31 of the particles chosen from preparations of IHN virus, SRCD virus, and OSV were 90 x 158 mμ, 90 x 159 mμ, and 91 x 181 mμ, respectively.

Bullet-shaped particles were also observed in electron micrographs of negatively stained, glutaraldehyde-fixed suspensions of OSV (see Figures 12, 13, and 14 (1)). The average dimension determined from 37 bullet-shaped particles was 98 x 166 mμ. Forty-six percent of these particles were 175 mμ long and 95 mμ in diameter. Thus, the sizes determined for bullet-shaped particles observed in fixed suspensions of OSV agree very closely in these two separate and independent investigations. For this and other reasons mentioned earlier,

these bullet-shaped particles are presumptively identified as the OSV virion.

VHS virus, which also infects salmonid fishes, i.e., rainbow trout, has been examined electron microscopically by Zwillenberg et al. (1965). Negatively stained and formalin-fixed suspensions of VHS contained bullet-shaped particles, considered to be the VHS virion, which had a morphology very similar to that observed for the presumptive OSV virion. The average dimensions of the negatively stained VHS virions were 70 x 180 mμ. Although the length of the VHS virion is similar to that found for the bullet-shaped particles of OSV, the diameter reported for VHS is about 25 mμ smaller.

Dimensions reported for the VHS virion by Zwillenberg et al. are similar to those reported for the bullet-shaped viruses, VSV and rabies virus. Hackett (1964) and Howatson and Whitmore (1962) determined the average dimensions of negatively stained VSV particles as 65 x 170 mμ and 68 x 175 mμ, respectively. Bullet-shaped particles in negatively stained suspensions of rabies virus were found to have mean dimensions of 75 x 180 mμ by Hummeler, Koprowski and Wiktor (1967) and Sokol et al. (1968).

Thus, it is possible that all RNA-, lipid-containing, bullet-shaped animal viruses (tentatively placed in the Rhabdovirus group) may have the same average dimensions, i.e., approximately 70 x 175 mμ (Fenner, 1968; Wilner, 1969). If this assumption is correct,

then the 98 mμ diameter determined for the bullet-shaped particles observed in glutaraldehyde-fixed OSV suspensions may be an artifact of specimen preparation. Greater particle diameters could have resulted from differential centrifugation, glutaraldehyde fixation, or drying of virus particles on specimen grids.

However, the 98 mμ average diameter of the presumed OSV virion may reflect actual physical differences between the OSV and the above mentioned Rhabdoviruses. This possibility is suggested by the fact that Amend and Chambers (1969), using a different virus fixative and a different cell line to propagate OSV, determined an average diameter of 91 mμ for the bullet-shaped particles of OSV.

An important facet of the electron microscope observations reported in this thesis, as well as by Amend and Chambers (1969), and by Zwillenberg et al. (1965) has been the inclusion of a virus-fixation step in specimen preparation techniques. A likely explanation for the use by Amend and Chambers of osmic acid for fixing OSV suspensions, and formalin fixation of VHS virus by Zwillenberg et al., is the unstable nature of these viruses. This instability of OSV may account for the fact that very few of the bullet-shaped particles, observed in glutaraldehyde-fixed virus suspensions, were found in negatively stained, unfixed suspensions of OSV (see Figures 14 (2), (3) and (4)). Instead of bullet-shaped particles being the most numerous type of particle in unfixed preparations, a particle

composed of two closely associated, roughly spherical (almost hexagonal) forms was the most abundant. Together, the two subunits formed a particle approximately 80 mμ in diameter and 160 mμ in length.

The differences between the appearance of glutaraldehyde-fixed and unfixed suspensions of OSV may provide an answer to the question why Wingfield (1968) proposed that the OSV virion had icosahedral symmetry and was between 91 and 145 mμ in diameter. In shadowed and negatively stained preparations of unfixed OSV, Wingfield observed particles which appeared to have hexagonal profiles. Such profiles suggest icosahedral symmetry (Horne and Wildy, 1963). It is difficult to know whether the particles considered by Wingfield to be the probable OSV virion were the same as the roughly spherical forms described above as the most numerous particles in unfixed OSV preparations. Nevertheless, the possibility exists that the OSV virion is bullet-shaped, and in both cases where unfixed OSV suspensions were employed, the specimen preparation techniques disrupted or distorted these particles.

Another interpretation of the discrepancy between the types of particles observed in fixed and unfixed OSV suspensions in this research and in that of Wingfield's, is that suspensions of OSV contain more than one type of virus particle. Although this possibility does not seem likely, additional research involving cloning of the virus, and

particle counts versus virus infectivity studies, must be performed before this factor can be ruled out.

SUMMARY AND CONCLUSIONS

1. Experiments were performed with Oregon sockeye salmon virus (OSV) propagated in either of two cell lines, SSE-30 and CHSE-214, derived from the embryonic tissues of sockeye and chinook salmon, respectively. Infectivity assays were performed by the end-point dilution method or the monolayer-plaque assay technique.
2. Partial purification of infectious culture medium was performed by differential centrifugation. The technique consisted of centrifuging infectious medium with a low-speed cycle (either 2,200 x g for 30 min, 8,700 x g for 15 min, or 19,600 x g for 15 min, depending upon the type of experiment), and subjecting the supernate from this step to a high-speed cycle of 55,000 x g for 60 min.
3. OSV suspensions exhibited a propensity for forming viral aggregates during differential centrifugation. In cases where a low-speed cycle of 2,200 x g for 30 min was employed, approximately 80% of the virus infectivity in concentrated suspensions of the high-speed pellet was sedimentable at 130 x g for 10 min. Evidence indicated that most viral aggregation was induced by pelleting OSV particles in the presence of host cell debris and serum proteins. Low-speed cycles of 8,700 x g for 19,600 x g for 15 min were incorporated into differential centrifugation procedures in an effort to remove more cellular debris prior to virus

pelleting during the high-speed cycle. Modifying the low-speed cycle did not significantly improve the percentage of virus recovery; however, suspended high-speed pellets were unaffected by centrifugation at 130 x g for 10 min.

4. A "complete" virus purification procedure involved differential centrifugation, treatment with RNase and DNase, and rate-zonal sucrose gradient centrifugation. Partially purified and nuclease-treated suspensions of OSV formed four visible bands when fractionated in 10 to 55 or 60% sucrose gradients. One band was located near the top of a gradient, two closely associated bands were in the middle, and a diffuse band was near the bottom of a gradient. Direct and indirect experimental evidence suggested that the two middle bands represented a heterogeneous virus band with the lower of the two bands containing infectious particles and the other consisting of noninfectious, incomplete virus particles. Aggregates of cell debris complexed with infectious virus were assumed to make up the lowest band in the gradients. The top band was presumed to contain mixtures of slow sedimenting cell materials and serum proteins with a small amount of complexed virus.

5. Of the virus infectivity in OSV suspensions subjected to rate-zonal sucrose gradient centrifugation, approximately 20 to 30% was detected in the virus band. A similar loss of infectivity (70 to 80%)

was encountered when the sucrose concentration in the pooled gradient fractions containing the virus band was reduced by dilution and/or dialysis.

6. The density of the OSV was 1.16 g/cm^3, as determined by isopycnic sucrose gradient centrifugation. Three peaks of virus infectivity were detected in gradient fractions. Percentages of virus infectivity in each peak, and the visible bands with which the peaks were associated were as follows: upper peak, 1%, top band; middle peak, 85%, two middle bands; lower peak, 6%, bottom band.

7. OSV suspensions, purified by the "complete" procedure and dialyzed exhaustively to eliminate sucrose, were treated with hot perchloric acid (PCA) in order to extract viral nucleic acid. The RNA and DNA concentrations in the PCA-extracts were estimated with the orcinol and diphenylamine tests. Concentrations of RNA were at least 14 times higher than those of DNA. Trace amounts of detected DNA were assumed to be caused by nonspecific reactions between diphenylamine reagents and viral proteins, or contamination of viral nucleic acid solutions with host cell DNA.

8. ^{32}P-labeled suspensions of OSV were subjected to differential centrifugation, RNase and DNase treatment, and rate-zonal sucrose gradient centrifugation. Close correspondence was

observed between infectivity and radioactivity detected in sucrose gradient fractions. Viral nucleic acid was extracted from the fractions representing the virus band by a procedure involving treatment with sodium dodecyl sulfate (SDS) and dimethylsulfoxide (DMSO), and extraction with buffer-saturated phenol at 4°C. Phenol was removed from aqueous phases by ether extraction. The dissolved viral nucleic acid precipitate had the following spectrophotometric properties: 260/230 mμ absorbance ratio, 0.76; 260/280 mμ absorbance ratio, 1.37. The spectral properties of viral nucleic acid solutions indicated that they contained relatively large amounts of protein.

9. Isopycnic cesium sulfate gradient centrifugation was used to band ^{32}P-labeled OSV nucleic acid. In two separate experiments, sharp peaks of radioactivity were detected at densities of 1.58 and 1.59 g/cm^3, respectively. No significant radioactivity was found in the region of the gradients where DNA would have banded. These data confirmed previous experimental results indicating that the OSV contains RNA. The low density of the viral RNA was attributed to nucleoprotein complexes.

10. An attempt to determine the sedimentation coefficient of ^{32}P-labeled OSV RNA was made by rate-zonal centrifugation of viral RNA in a 10 to 30% glycerol gradient. Radioactivity was distributed in a broad peak with a maximum value at 26 S and a pronounced

shoulder at 37 S. The apparent heterogeneous nature of OSV RNA was possibly related to proteins associated with RNA molecules, breakdown of RNA by nucleases, or contamination by nonviral RNA. A sedimentation coefficient of 37 S, determined for a major fraction of the viral RNA molecules, approximates similar values reported for VSV and rabies virus RNA.

11. Fractions from the glycerol gradient used to measure the sedimentation coefficient of OSV RNA were divided into equal volumes. One set of fractions was used to determine the distribution of the viral RNA, and the other set was treated with RNase. The amount of trichloroacetic acid-precipitable radioactivity in each set of fractions was measured. RNase treatment reduced all ^{32}P-radioactivity by 85 to 97%, indicating that OSV RNA is single stranded.

12. Anion exchange chromatography was employed to analyze the nucleotide composition of ^{32}P-labeled OSV RNA. RNA in the most radioactive fraction, i.e. the RNA band, from one cesium sulfate gradient was subjected to alkaline hydrolysis; and three separate analyses were performed. The average percentage composition values were cytidylic acid, 25.8 ± 0.6%; adenylic acid, 23.0 ± 0.8%; uridylic acid, 27.7 ± 0.6%; and guanylic acid, 23.4 ± 0.4%. The nucleotide composition of OSV RNA also indicates that the viral RNA molecules are single stranded.

13. Rabbit-immune sera were produced against OSV, IHN virus and SRCD virus. Differentially centrifuged virus suspensions, containing 5.0×10^8 to 2.0×10^9 $TCID_{50}$/ml, were injected, undiluted or emulsified with complete Freund's adjuvant, into rabbits. Two injection series per rabbit were administered 14 days apart. The total virus infectivity injected into rabbits was proportional to the 50% plaque neutralization end points obtained with the respective antisera. End points of IHN virus, OSV, and SRCD virus antisera versus their homologous virus were approximately 1/160, 1/340, and 1/1600, respectively.

14. Cross plaque neutralization tests were performed with each antiserum versus OSV, IHN virus and SRCD virus. The 50% plaque neutralization end points determined for OSV and IHN virus with all three antisera did not differ significantly. End points measured for SRCD virus were consistently about two-fold higher than those determined for OSV and IHN virus, regardless of whether SRCD virus was homologous or heterologous to the serum being tested. Antigenically, all three viruses were found to be related, with OSV and IHN virus being indistinguishable.

15. An electron microscope was employed to examine differentially centrifuged suspensions of OSV. In some cases, partially purified suspensions were fixed with glutaraldehyde. Fixed and unfixed specimens were stained with phosphotungstic acid. The

most numerous type of particle in fixed preparations was bullet-shaped. The average dimensions of 37 well stained bullet-shaped particles were a length of 166 mμ and a diameter of 98 mμ. These particles consisted of a striated cylinder (24-25 striations per cylinder) surrounded on the sides and rounded end by an outer coat (10 to 14 mμ thick). A much smaller proportion of bullet-shaped particles was observed in unfixed OSV preparations. The most abundant type of particle in unfixed suspensions consisted of two roughly spherical, closely associated particles. Each of the two spherical forms were about 80 mμ in diameter, and together they formed a particle 80 x 160 mμ. The discrepancy between the appearance of fixed and unfixed OSV suspensions was not experimentally resolved; however, the fragility of the OSV was suspected as the most likely cause for the reduced numbers of bullet-shaped particles in unfixed preparations.

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