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Title: Purification and Characterization of Avian Encephalomyelitis Virus

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Nobakazu Matsumoto

Avian encephalomyelitis virus-infected chick embryo homogenate was precipitated with polyethyleneglycol 6000 (PEG), extracted with fluorocarbon, concentrated by ultrafiltration, and purified by isopycnic gradient centrifugation. The physical and chemical properties of the purified virus were studied. The examination of purified avian encephalomyelitis virus (AEV) with electron microscopy using negative stain revealed virus particles which were icosahedral in shape, lacked an envelope, and had a mean diameter of 26 $\pm$ 3 nm. It was suggested that the viral capsid was composed of 12 capsomers located at the vertices of an icosahedron. The virus was tentatively given a triangulation number of one, and included in the icosahedral class P = 1. The sedimentation coefficient ($S_{20,w}$) of AEV was determined to be 148 $\pm$ 3 S. The bouyant density of the virus in CsCl was determined to be 1.31 to 1.32 g/ml by infectivity assay following isopycnic gradient centrifugation. The infectivity titer of the virus was reduced by 1.2 $\log_{10}$ after heating at 56 C for one hour. The virus

was stabilized to the effect of heat by 1 M $MgCl_2$. The virus was resistant to treatment with chloroform and trypsin. Attempts to discern the type of nucleic acid in the virus genome were inconclusive.

Purification and Characterization of Avian Encephalomyelitis Virus

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in charge of major

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Dean, School of Veterinary Medicine

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INTRODUCTION

The infectious agent of avian encephalomyelitis (AE) was demonstrated to be a virus by Jones (1934). Butterfield et al. (1969) used a suspension of infected chick brain material in characterization studies of avian encephalomyelitis virus (AEV). They determined that the virus had a bouyant density in CsCl of 1.33 g/ml, and that it was resistant to treatment with chloroform, pepsin, and deoxyribonuclease. The virus was slightly affected by ribonuclease, and it was resistant to inactivation by heat and acid pH. However, this work was performed on nonpurified samples of virus. Matsumoto and Murphy (1977) developed a procedure for the partial purification of the virus with polyethyleneglycol 6000 (PEG) and fluorocarbon. The present study was directed towards the purification of AEV, and subsequent physical and chemical characterization of the purified virus. The information concerning the purification and concentration of AEV is valuable to the efforts directed towards the development of a convenient diagnostic test for AE infection.

LITERATURE REVIEW

Avian encephalomyelitis was first described by E. E. Jones in 1932 as a nervous disorder of one to six-week-old chicks which was associated with rapid tremors of the head and neck, as well as ataxia of the affected birds. The increased number of observations of the disorder in New England chicken flocks prompted Jones (1934) to name the disease epidemic tremor of chickens. The disorder was tentatively classed as a viral disease at this time since the disease was successfully passed to susceptible chickens by intracerebral inoculation of bacteriologically sterile filtrates of brain and spinal cord suspensions from infected chickens (Jones, 1934). Based on histological lesions, the disease was later renamed infectious avian encephalomyelitis (Van Roekel et al., 1938).

The first recognizable symptoms of the disease in natural outbreaks are seen in chicks of one to two weeks of age (Luginbuhl and Helmboldt, 1972). The eyes of the chicks take on a dull expression, and progressive ataxia soon follows. Tremors of the head and neck may occur. The final stages of the disease are inanition, prostration, and death. Masses of infiltrating lymphocytes in the muscularis of the ventriculus form the only gross lesion which can be discerned in the infected chick. The disease in adult chickens can only be detected by a decrease in egg production, serological testing, and the occurrence of the disease in the progeny.

The histopathological changes in the infected chick occur in the central nervous system and the viscera. The lesions in the central

nervous system are a ganglionitis of the dorsal root ganglia and a disseminated, nonpurulent encephalomyelitis (Luginbuhl and Helmboldt, 1972). A perivascular cuffing of small lymphocytes often occurs in most regions of the brain and spinal cord. Central chromatolysis of the neurons in the nuclei of the brain stem constitutes an additional lesion which is of diagnostic value. Lesions in the spinal cord are similar to those in the brain. Visceral lesions consist of hyperplastic lymphocytic aggregates which are randomly located throughout the body.

Transmission of the disease can occur through direct or indirect contact (Calnek et al., 1960). Susceptible chickens which were placed in a colony house which previously contained AEV-infected birds developed positive serology within three to seven weeks. Chicks could be infected via the intestinal route to the exclusion of the respiratory tract. The administration of AEV in the drinking water of susceptible breeders resulted in a syndrome which was similar to that obtained in natural outbreaks (decreased egg production, lowered hatchability, and egg transmission). The syndrome occurred in 6 to 13 days following inoculation. The presence of AEV in the feces was demonstrated during this period. Chicks can be infected either through egg transmission or contact with infected chicks. Chicks which are infected through the egg (vertical transmission) manifest signs of the disease in one to seven days of age, whereas chicks infected through contact (horizontal transmission) show signs of the disease 11 to 16 days after exposure.

All of the isolates of AEV have been enterotropic, and thus far no serologic differences have been detected among them (Luginbuhl and Helmboldt, 1972). A distinction should be made between the natural strains (field isolates) and those that have been embryo-adapted. The natural strains infect young chicks (less than four-weeks-old) readily via the oral route and the virus is shed in the feces. Many field isolates are nonpathogenic for embryos. Field isolates of AEV were adapted to embryos by serial passage of infected brain material in embryos (Sumner et al., 1957). The embryo-adapted strain produces a muscular dystrophy and paralysis of voluntary muscles. In contrast to the natural strains, embryo-adapted strains do not infect young chicks via the oral route although they do infect after parenteral inoculation. The embryo-adapted strains are routinely employed for the neutralization test to detect anti-AEV antibody and for the vaccination of pullets in drinking water as described below.

The general sites of AEV multiplication were studied by several workers. Burke et al. (1965) inoculated an embryo-adapted strain of AEV, Van Roekel (VR) strain, into five to six-day-old embryos via the yolk sac. The virus was found in chorioallantoic membrane, allantoic fluid, yolk with yolk sac, viscera, and brain material at various times after the inoculation. The highest virus yields were found in the brain and viscera at nine days postinoculation. More recently Ikeda et al. (1976,a) studied the multiplication of VR strain in two-day-old chicks. The virus titer in chicks showing clinical signs was highest after seven to nine days in the brain, heart, liver, spleen,

and pancreas. Persistent presence of the virus was demonstrated in the pancreas, brain, and eyeball as long as 21 days postinoculation. Similar experiments (Ikeda et al., 1976,b) showed the detection of the virus in low titers mainly in the central nervous system for up to 17 days in 40-day-old chickens and the presence of the virus only in the inoculated muscle sites for a period of seven days in 110-day-old chickens. Parenteral administration of VR strain in laying hens resulted in the detection of the virus in the central nervous system for 11 days, but only in low titers. The virus disappeared rapidly from other organs. In sharp contrast to the above observation, when a field isolate of AEV was inoculated into laying hens, either by oral or parenteral route, the virus was detected in high titers in the central nervous system and parenchymal organs for more than three weeks (Ikeda et al., 1976,c). The virus titer dropped rapidly after initially high levels in the digestive tract and the ovarian follicle.

Several reports in the literature have claimed successful propagation of AEV in cell cultures, but always in low titers and without observable cytopathic effects, plaques, or inclusion bodies. The virus was grown in chicken embryo neuroglial cell culture (Mancini and Yates, 1967), chicken embryo fibroblastic cell culture (Mancini and Yates, 1968), and in chicken embryo kidney cell culture (Mancini and Yates, 1968). Titrations of cell culture fluids from chicken pancreatic cell cultures which were inoculated with AEV were $10^{2.9-3.7}$ with the maximum yield occurring eight days postinoculation (Kodama et al., 1975).

Birds recovering from natural and experimental infections of

AEV develop a level of immunity due to the presence of antibodies capable of neutralizing the virus (Sumner et al., 1957). Immunity to the disease is passively transferred to progeny from immune hens. The passive immunity protected the chicks for at least eight to ten weeks of age (Calnek et al., 1961). Serum neutralization tests have been used to test the immunity levels of flocks against AEV (Calnek and Jehnich, 1959). The sera of unvaccinated, normal chickens tested had a mean neutralization index (NI) of 0.14. The birds were vaccinated by intramuscular (IM) or wing-web (WW) inoculation. Neutralization indices of 1.3 to 3.4 were detected four weeks postinoculation. Birds with NI of this level resisted challenge with intracerebral inoculation with 10,000 fifty percent embryo-infective dose (EID_{50}) units of AEV. Neutralization properties were found in the sera of partridges, pheasants, and turkeys, but not in sera from finches, sparrows, starlings, jackdaws, rooks, doves, pigeons, or ducks (Van Steenis, 1971). Virus neutralizing substances were found in adult serum, yolk, and serum of progeny from chicken flocks which allowed only reduced growth of AEV in inoculated embryos (Sumner et al., 1957).

The failure of eggs from immune hens to support the growth of AEV was used to survey the prevalence of AE in chicken flocks. Evidence of the disease was found throughout the United States and Canada (Taylor and Schelling, 1960). Turkey flocks were similarly tested and 61% of the flocks tested were immune. An additional 11.3% were recently exposed or had declining protection (Deshmukh et al.,

1971).

The roles of humoral and cellular immunities in the pathogenesis of AE were examined by inoculating bursectomized (to abolish antibody formation) and thymectomized (to abolish cellular mechanisms of immunity) chicks of 1, 8, or 28 days of age (Cheville, 1970). The natural pattern of infection was not altered in thymectomized chicks. Bursectomy was followed by severe paralysis in 17 days regardless of the age of the chicks.

Immunization procedures have been the most closely studied aspect of the disease. Vaccination by the IM route provided the best protection but it also resulted in the greatest incidence of clinical infections (Schaaf, 1958). The immune response to WW inoculation developed more slowly than that following IM inoculation. An inactivated vaccine was prepared with beta-propiolactone which contained 6.25% aluminum hydroxide as adjuvant (Schaaf, 1959). Spray, oral, WW, and IM methods of inoculation were compared in six and one-half to eight-week-old chickens. Up to 40% pathogenicity was observed following inoculation by all methods except oral administration. Good serological responses were obtained after WW or IM injection of 10^3 EID_{50} units of AEV. Oral inoculation required 10^6 EID_{50} units to be effective (Calnek and Jehnich, 1959). The recommended period for administration of oral vaccine (live field virus added to the drinking water) is between the tenth week of age and three weeks prior to egg production to avoid egg transmission of the virus and interference by passive immunity in birds under ten weeks of age (Calnek et al., 1961). A

lyophilized vaccine using a less virulent strain of AEV was developed which was safe to use with fully susceptible birds of one week of age or older (Lawson and Gregg, 1969). A freeze-dried AE vaccine administered in the drinking water or by WW inoculation was shown to be very effective (Polewaczyk et al., 1972).

Attempts to develop a rapid and easily performed diagnostic test for AE have met with little success. Hemagglutination tests which used erythrocytes from fowl, human group A, turkey, magpie, pigeon, herring gull, horse, and sheep were unsuccessful (Halpin, 1966). Complement-fixation tests with homologous immune chicken serum showed specific but low complement-fixation activity (Sato et al., 1969). Fluorescent antibody technique has been successful in detecting antigen in young chicks but not in mature chickens or in cell cultures (Miyamae, 1974; Kodama et al., 1975). Indirect fluorescent antibody technique using rabbit anti-chicken gamma globulin was shown to be more sensitive than the virus neutralization test (Choi and Miura, 1972). Fluorescent-blocking antibody tests show that the antibodies associated with this reaction are transient and do not persist in the serum as long as the virus neutralization antibodies (Davis and Lukert, 1971). In spite of considerable effort, the embryo susceptibility test and the virus neutralization test are the only means of monitoring chicken flocks for AEV. Since these tests are time consuming and uneconomical, efforts to develop a more satisfactory test continue.

Electron microscopy was used to examine a sample prepared by

resuspending in a small volume the pelleted material from a chloroform extraction of infected chick brain material (Krauss and Ueberschar, 1966). Negative staining of the preparation revealed particles which were icosahedral in shape and lacked an envelope. A mean diameter of 23.3 nm with a standard deviation of 2 nm was determined for the virus particles. It was proposed that the virion possessed 12 morphological units of a compact type.

Physical and chemical tests performed on suspensions of infected chick brain were used to characterize the virus (Butterfield et al., 1969). The virus was resistant to treatment of deoxyribonuclease (DNase) while it was slightly affected by ribonuclease (RNase). The virus lacked essential lipids since it was resistant to treatment with chloroform, and the virus was stable at pH 2.8 for three hours. It was determined that the virus was heat resistant (for the most part) and it was stabilized to the effect of heat by magnesium cations. The virus had a specific gravity of 1.33 in CsCl, and a particle size of 16.5 to 25 nm as determined by filtration. Attempts to propagate AEV in cell cultures of bovine kidney, chick kidney, and chick fibroblast were unsuccessful. On the basis of the above results, AEV was classified as a member of the genus Enterovirus in the family Picornaviridae. The virus, however, has never been purified to date and, therefore, the classification by Butterfield et al. (1969) has not been widely accepted.

MATERIALS AND METHODS

Embryos

The stock virus was propagated in type I specific-pathogen-free chicken embryos (H & N Incorporated). Infectivity tests and the preparation of large quantities of infected embryos were accomplished with embryonating eggs which were obtained from the Department of Poultry Science, Oregon State University. White Leghorn breeders, vaccinated against fowl pox only, were the source of the Poultry Science embryos. The embryos from both sources showed no significant variation in susceptibility to infection with AEV.

Virus

Embryo-adapted AEV (Sumner et al., 1957) was inoculated into six-day-old embryos via the yolk sac in 0.1 ml amounts which corresponded to 1000 EID_{50} units/ml. The diluent used for all virus dilutions was Hanks' balanced salt solution with 0.5% lactalbumin hydrolysate (GIBCO) and antibiotics (50 I. U. penicillin and 50 μg streptomycin per ml). Following inoculation, the embryos were incubated for ten days. At this time, embryos showing typical AE lesions (Luginbuhl and Helmboldt, 1972) were aseptically removed and the beaks and shanks discarded. The remaining tissue was homogenized in a 20% suspension in a Waring blender. The homogenate was treated with three cycles of freezing and thawing, and centrifuged at 1,000 x g for 15 minutes. The supernatant was collected and frozen at -70 C.

Infectivity measurement

Tests of infectivity were done as described by Calnek and Jehnich (1959). The fifty percent embryo-infective dose (EID_{50}) was calculated by the method of Reed and Muench (1938).

Precipitation with PEG

The crude virus harvest was precipitated by the dropwise addition of sterile 20% polyethyleneglycol 6000 (PEG) (Carbowax; Fisher Scientific Company) solution in the tris-HCl pH 7.6 buffer containing 0.5 M NaCl (tris saline) to a final concentration of 4% (w/v) (Matsumoto and Murphy, 1977). The mixture was stirred for 90 minutes at 4 C before centrifugation at 5,900 x g for 15 minutes. The supernatant was discarded and the precipitate was washed once with 4% PEG solution. The precipitate was resuspended to its original volume in tris saline. The suspension was homogenized in a Waring blender and then used for the extraction with fluorocarbon.

Fluorocarbon extraction

Equal amounts of trichlorotrifluoroethane (TTE) (Freon TF; du Pont de Nemours and Company) and the virus sample were mixed in a Waring blender for three minutes. The mixture was centrifuged at 2,000 x g for 30 minutes, and the aqueous layer was collected (Matsumoto and Murphy, 1977).

Ultrafiltration

The crude virus suspension was concentrated in an ultrafiltration cell (Amicon model 52) using a membrane of 1.8 nm apparent pore size (Amicon PM 10). A liquid reservoir (Amicon RS 4), pressurized with 25 psi of nitrogen gas, continuously supplied the solution to the cell. The ultrafiltration process was continued until the suspension was concentrated by approximately twenty-fold.

Sonication

The ultrafiltration concentrate was treated for 15 seconds at 20% capacity (high power) with a sonicator (Bronwill Scientific Biosonik IV). After sonication, the concentrate was passed through a cellulose filter of 0.22μm retention size (Millipore).

Ultracentrifugation

Isopycnic gradient centrifugation (Brakke, 1960) was used to purify and concentrate the virus. The virus sample was added to a cesium chloride solution (density = 1.34), prepared by dissolving CsCl in tris-buffered saline, and centrifuged for 18 hours at 85,000 x g in a swinging bucket rotor (Beckman SW 50.1) at 4 C. The cellulose nitrate centrifuge tubes were punctured at the bottom and ten-drop fractions were collected. The fractions containing virus were pooled and dialyzed against tris-buffered saline at 4 C. This virus was used for testing sensitivity to chloroform, sensitivity to trypsin, acid pH sensitivity, heat inactivation and stabilization by 1 M magnesium

cations, and as inoculant for cell culture experiments. For electron microscopy, determination of the sedimentation coefficient, and phenol extraction of nucleic acid, the virus was pelleted by layering 2 ml of the preparation onto 3 ml of 20% glycerol in tris-buffered saline containing 0.001 M EDTA, and centrifuging at 150,000 x g for one hour at 4 C. The pellet was resuspended in a small amount of tris-buffered saline.

Density

The density of each fraction was determined by weighing a known volume of the fraction in a 500 μl Lang Levy pipette. The densities were also determined by assaying the optical density of the fraction with a refractometer (Bausch and Lomb ABBE-3L). Each fraction was assayed for infectivity in embryonating eggs as previously described.

Sedimentation coefficient

The virus sample was repeatedly dialyzed against tris-buffered saline. Moving boundary sedimentation experiments were done in a Spinco model E centrifuge with an AN-D rotor. The ultraviolet absorption was measured at 265 nm every 4 minutes. The distance (r) of the boundary from the center of rotation was measured of a minimum of 10 interval plots. The values ln r were graphed versus time, and the deviations were calculated. Values with notable deviations were arbitrarily eliminated. The sedimentation coefficient ($S_{20,w}$) was corrected for buffer viscosity and temperature.

Electron microscopy

Virus preparations were mounted on formvar films (0.25% in ethylene dichloride) supported on 400-mesh copper specimen grids (Athene). The preparations were examined in a Phillips EM 300 electron microscope at 80 kilovolts and 20 microamps. Electron images were recorded on Kodak Electron Image Plates. Virus preparations were shadowed with chrome (99.99%) in a vacuum evaporator (Varian-Mikros model VE-10). The evaporating source was 117 $\pm$ 2 mm from the specimen grid, and it was elevated 15 $\pm$ 2^{o} above the plane of the grid. The virus particles were negative stained by mixing the virus sample with an equal volume of 2% sodium phosphotungstate (pH 7.0). Bovine catalase crystals (Tousimis Research Corporation) were added as an internal standard for measurement of the virus particles (Wrigley, 1969). The mixture was applied with a glass nebulizer (DeVilbiss no. 40). All measurements of virus particles and bovine catalase crystals were made on the original plates with dial calipers.

Sensitivity to trypsin

Fractions which corresponded to densities of 1.31 to 1.32 g/ml were pooled and dialyzed against tris-buffered saline in the cold for two hours. Equal volumes of virus sample and trypsin (Difco 1:250) at concentrations of 1, 0.5, 0.25, and 0.125 percent were mixed and incubated at 37 C in a water bath for 60 minutes according to the method of Kurogi et al. (1976). Tris saline was added to one virus sample as a control. Soybean trypsin inhibitor (Worthington) was added

at the end of the incubation period, and the mixtures were allowed to stand at room temperature for ten minutes before they were placed in an ice-water bath. Samples from each mixture were diluted and inoculated into embryonating eggs for determination of the virus infectivity.

Acid pH sensitivity

The sensitivity of AEV to acid pH was tested with slight modifications to the method of Ketler et al. (1962). Two solutions of HLH were adjusted to pH 3.0 and 7.1 respectively. The solutions were passed through cellulose filters of 0.22μm retention size. Virus samples were diluted 1:10 in both pH solutions and allowed to stand at room temperature for 60 minutes. Both mixtures were tested for infectivity.

Sensitivity to chloroform

The susceptibility of AEV to a lipid solvent was tested by adding 0.5 ml of chloroform to 1 ml of purified virus sample. One-half ml of HLH was added to a second virus sample as a control. Both tubes were shaken for 10 minutes at room temperature, and then centrifuged at 500 x g for five minutes. The aqueous phase was collected and titrated in embryos.

Heat inactivation and stabilization by 1 M $MgCl_2$

A purified virus preparation was diluted 1:10 in sterile

deionized double-distilled water. Three samples of 1 ml were distributed to test tubes. One ml of 2 M $MgCl_2$ was added to one tube and equal amounts of sterile deionized double-distilled water were added to the remaining test tubes. The test tubes were sealed and the tube containing $MgCl_2$ and one control tube were incubated at 56 C for one hour. The other control tube was stored at 4 C for the same period. All samples were titrated for virus infectivity.

Type of nucleic acid

Ribonuclease I (RNase; Worthington Biochemical Corporation cat. no. 5679) was diluted in 0.1 M NaCl to a final concentration of 9.8 μg/ml. Deoxyribonuclease I (DNase; Worthington Biochemical Corporation cat. no. 2172) was rehydrated with 0.9% NaCl and diluted to a final concentration of 10μg/ml with a solution of 0.01 M $MgCl_2$ and 0.01 M NaCl (pH 6.5). Virus samples were added to equal volumes of RNase, DNase, and tris-buffered saline (pH 7.6), and incubated for 30 minutes at 37 C. The infectivity of each mixture was assayed in embryonating eggs. A phenol extraction was performed on pelleted virus according to the following procedure. The virus sample was mixed with an equal volume of liquified phenol and shaken hard for five minutes at room temperature. The mixture was centrifuged at 10,000 x g for five minutes. The aqueous phase was collected and transferred to another centrifuge tube. Sufficient 3 M sodium acetate and 0.001 M EDTA solution was added to give a sodium concentration of 0.15 M. Two and one-half volumes of 95% ethanol were added to the aqueous phase.

The solution was centrifuged at 17,000 x g for 40 minutes at 4 C. The supernatant was discarded and the pellet was dissolved in a small volume of 0.01 M tris buffer and 0.001 M EDTA, and stored frozen until use. The final product of the extraction was divided into three portions and treated as described above with RNase and DNase. The acridine orange fluorochrome procedure as set forth by Rovozzo and Burke (1973) was used on air-dried virus samples. The virus samples were fixed in a graded series of alcohol dips. One sample was immersed in DNase (0.01% in 0.025 M veronal buffer plus 0.003 M $MgSO_4$), another sample was immersed in RNase (0.05% in distilled water, pH 7), and the third sample was placed in a solution of 0.02 M citric acid in 25% methanol and 0.04 M sodium phosphate (dibasic) in 25% methanol. All samples were incubated at 37 C for 30 minutes, and processed as described in the procedure. A diphenylamine colorimetric method for determination of DNA and an orcinol method for detection of RNA (Hatcher and Goldstein, 1969) were used to test the product of a phenol extraction of purified virus.

Preparation of chick embryo fibroblast cell culture

An eleven-day-old chicken embryo was aseptically removed from an egg and placed in a sterile glass Petri dish. The head, major bones, and viscera were removed and discarded. The tissue was finely minced with scissors. The minced tissue was removed to a flask in 2 ml of 2.5% trypsin, and gently agitated at 37 C for ten minutes. The larger tissue fragments were allowed to settle and the supernatant

was discarded. Two ml of 0.125% trypsin were added to the flask which was gently agitated at 37 C for ten minutes. The supernatant was removed and placed in an ice bath. One ml of fetal bovine serum (FBS) containing medium (Dulbecco's Modified Eagle Medium; DME) was added to the supernatant to inhibit the action of residual trypsin. The trypsinization step was repeated. The supernatants were pooled and centrifuged for four minutes at 150 x g. The cell suspension was removed from the packed debris. To determine the viable count a sample of the cell suspension was diluted 1:10 in DME (total volume = 1 ml) and stained with two drops of trypan blue. The cells were plated at a concentration of 1×10^6 cells/ml in 25 cm^2 flasks. Five ml of DME were added to each flask. The medium was changed every third day while the cells were growing to confluency.

Inoculation of cell culture with AEV

Dialyzed fractions of densities between 1.31 and 1.32 g/ml, from equilibrium centrifugation of crude virus concentrate, were pooled and diluted 1:10 in fetal bovine serum. The solution was passed through a cellulose filter of 0.22μm retention size (Millipore). In addition, ultrafiltration concentrate was passed through a similar filter of 0.45μm pore size and diluted 1:10 in DME containing Polybrene (5μg/ml; Sigma). The virus was added in 0.2 ml amounts per 25 cm^2 flask and allowed to adsorb for one hour at 37 C. Five ml of DME containing gentamycin (60μg/ml) were added to each flask. Forty-eight hours postinoculation the medium was removed from each flask and

replaced with five ml of fresh DME and FBS which contained 20 Ci/ml of (^{3}H)uridine (Uridine (S,6-^{3}H); ICN Pharmaceuticals, Incorporated). The cell culture fluid was harvested after 24 hours.

Liquid scintillation

Cell culture fluids were harvested and centrifuged at 150,000 x g for one hour at 4 C. The pellet was resuspended in 0.25 ml of tris saline containing 0.001 M EDTA. The suspension was layered onto a CsCl solution (density = 1.34) and centrifuged at 85,000 x g for 18 hours at 4 C. Ten-drop fractions were collected and the densities were determined using the refractive indices of CsCl. One ml of 10% trichloroacetic acid was added to each fraction. The fractions were allowed to stand for 15 minutes. One ml of 5% trichloroacetic acid with 0.01 M sodium pyrophosphate was added to each fraction. The fractions were filtered through reeve angel glass fiber filters (Whatman Incorporated). The tubes were washed six times with the 5% TCA solution. The filters were washed once with 95% ethanol. The filters were removed and dried under a lamp. The filters were placed in scintillation grade toluene (Scintrex reagent; J. T. Baker) which contained Omnifluor (15.14 g Omnifluor/ gallon of toluene). Five minute counts were taken in a liquid scintillation counter (Beckman LS 100).

RESULTS

Determination of density

The crude virus harvest was precipitated with PEG. The precipitate was resuspended and homogenized in the original volume of tris-buffered saline. The virus suspension was extracted with TTE, and the aqueous phase was collected and concentrated twenty-fold by ultrafiltration. The ultrafiltration concentrate was sonicated and filtered. The virus concentrate was layered onto a CsCl solution (density = 1.34 g/ml) and centrifuged at 85,000 x g for 18 hours. The centrifuge tubes were punctured at the bottom and ten-drop fractions were collected. The density of each fraction was determined.

To determine the bouyant density in CsCl of AEV, 0.1 ml of a 1:10,000 dilution of each fraction was inoculated into six-day-old embryos via the yolk sac. The embryos were removed and examined for typical AE lesions ten days postinoculation. Maximum infectivity was associated with two fractions of densities 1.31 to 1.32 g/ml (Table 1). An additional dilution failed to differentiate the two fractions since each was equally infective (60% at 10^{-5}/0.1 ml). In addition to the major peak, smaller peaks (Figures 1a and 2) were observed at densities of 1.40 and 1.36 g/ml.

Sedimentation coefficient

Two virus samples from separate purification procedures were analyzed in moving boundary experiments to derive a sedimentation co-

Table 1. Infectivity test results of fractions collected after equilibrium centrifugation in CsCl. Embryos were graded as to the presence and severity of typical AE lesions. Lesions: 0, no apparent lesions; 1, partial movement but some deformity of legs; 2, ataxia and deformity but normal size; 3, ataxia, deformity, and emaciation; D, embryo was dead.

Fraction number	Density	Lesions	Fraction infected	Percent infected
1	1.405	0, 0, 2, 0, 0	1/5	20
2	1.397	0, 0, 0, 2, 2	2/5	40
3	1.390	0, 0, 0, 0, 0	0/5	0
4	1.382	0, 0, 0, 0, 0	0/5	0
5	1.374	0, 0, 0, 0, 0	0/5	0
6	1.366	0, D, 0, 0, 0	0/4	0
7	1.359	2, 0, 2, 0, 2	3/5	60
8	1.350	0, 0, 2, 0, 0	1/5	20
9	1.343	0, 0, 0, 0, 0	0/5	0
10	1.335	2, D, 0, 0, 0	1/4	25
11	1.328	0, 0, 0, 0, D	0/4	0
12	1.320	3, 3, 2, 3, 3	5/5	100
13	1.312	2, 3, 3, 2, 2	5/5	100
14	1.304	1, 3, 2, 0, 1	4/5	80
15	1.296	0, 2, 2, 0, 2	3/5	60
16	1.288	0, 0, 2, 2, 0	2/5	40
17	1.281	0, 0, 2, 2, 0	2/5	40

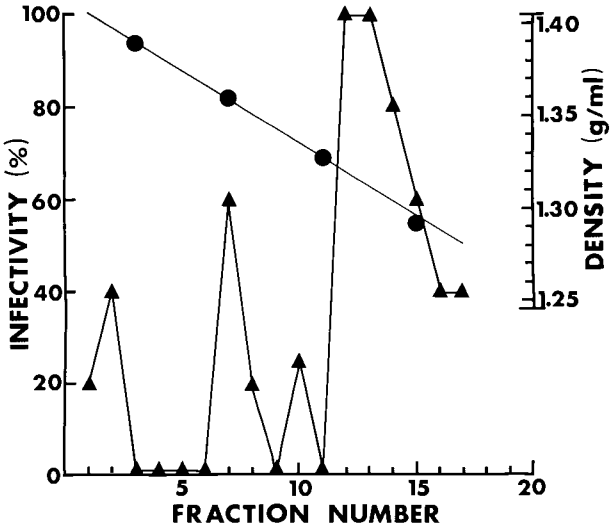

Figure 1 a. Equilibrium centrifugation of AEV in CsCl. Virus sample was centrifuged at 85,000 x g for 18 hours. Ten-drop fractions were collected, and the densities determined by weight and refractive index of CsCl. Infectivity of the fractions was determined by inoculation of six to seven-day-old embryos via the yolk sac with 1:10,000 dilutions of the respective fractions.

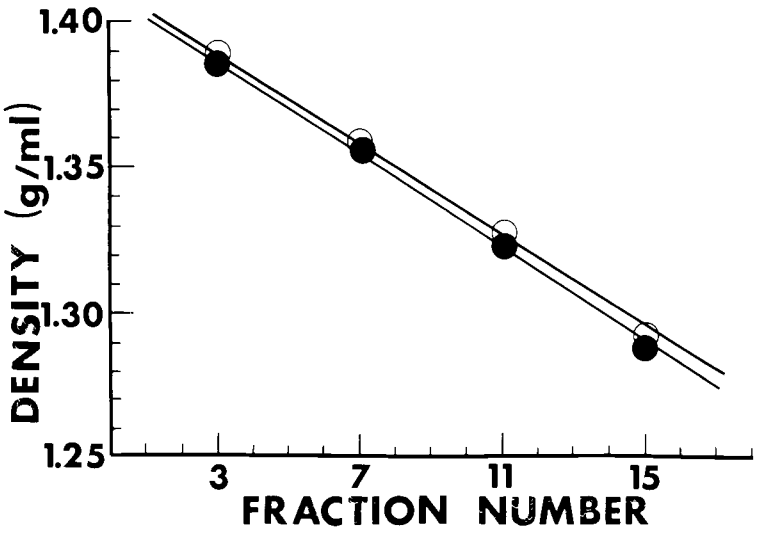

Figure 1 b. The comparison of density values derived from the refractive index of CsCl or weight of selected fractions following equilibrium centrifugation. Refractive index, ●—●; weight, O—O.

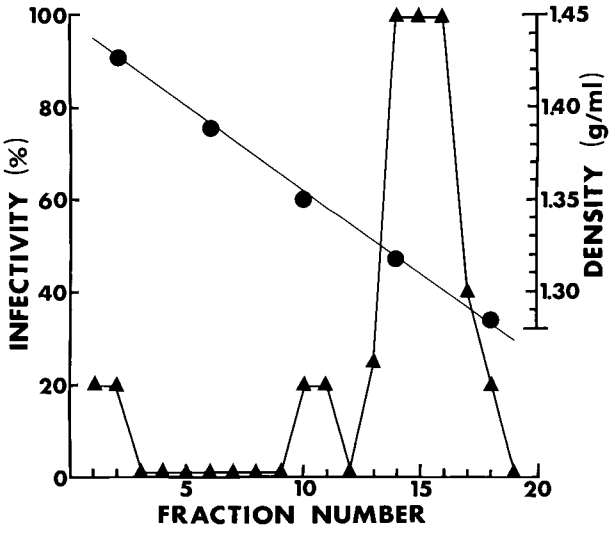

Figure 2. Equilibrium centrifugation of AEV in CsCl.

Table 2. Distances of the boundary from the center of rotation at fixed intervals as determined by optical absorption at 265 nm in moving boundary experiments with two purified AEV samples.

Experiment 1				Experiment 2			
Plot no.	Time (sec)	ln r	Deviation	Plot no.	Time (sec)	ln r	Deviation
1	55	1.781692	.000466	1	27	1.895416	-0.000256
2	533	1.792475	.001018	2	265	1.902496	.001521
3	1011	1.801484	-0.000213	3	504	1.906616	.000320
4	1488	1.810906	-0.001029	4	742	1.911612	.000000
5	1966	1.821543	-0.000622	5	981	1.915991	-0.000940
6	2443	1.831665	-0.000732	6	1220	1.921677	-0.000565
7	2921	1.839610	-0.002646	7	1458	1.926379	-0.001180
8	3398	1.853344	.000490	8	1935	1.938471	.000295
9	3875	1.863463	.000378	9	2174	1.942157	-0.001341
10	4352	1.873559	.000243	10	2412	1.950133	.001339

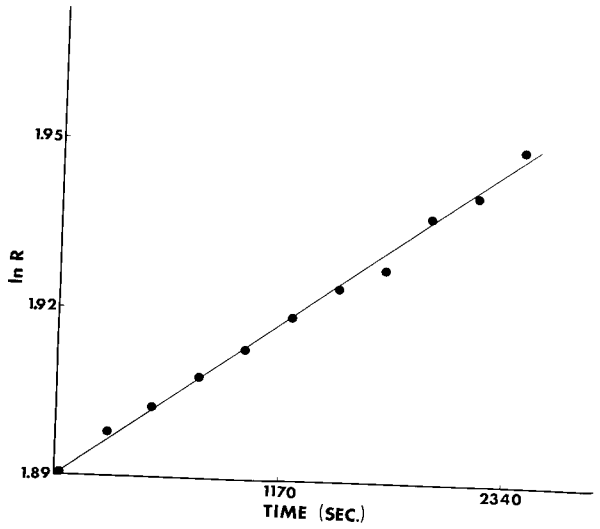

Figure 3. The relationship of the individual measurements to the plot of ln r versus time, as determined in moving boundary experiments with AEV.

efficient for AEV. The $S_{20,w}$ of the virus which were calculated from two experiments (Table 2) were 144.98 and 151.85 S. An arithmetic mean of the values, 148.43, was calculated to more clearly represent the sedimentation coefficient of the virus under the experimental conditions. A graph (Figure 3) is shown to illustrate the relationship of the individual measurements to the linear plot of ln r versus time.

Electron microscopy

The general shape and size of the virus particles can be discerned from the micrographs showing shadow-casting of the particles with chrome (Figures 4 and 5). These micrographs also attest to the purity of the sample. Purified virus samples were negative stained with sodium phosphotungstate. Bovine catalase crystals were added with the stain to act as an internal standard for measurement of the virus (Figure 6). All measurements were done on the original photographic plates. Twenty particles were measured, and the mean size was determined by comparison with the regular lattice spacing of the catalase crystals. The mean size of the measured virions was 26 ± 3 nm. The particles of AEV were icosahedral in shape. The majority of viral particles failed to show any surface structure. No single set of conditions was found which definitively showed the surface substructure of every virus particle examined. However, the capsomers could be clearly discerned on some particles (Figure 7). The arrangement of the capsomers strongly suggested a 12 subunit structure of the

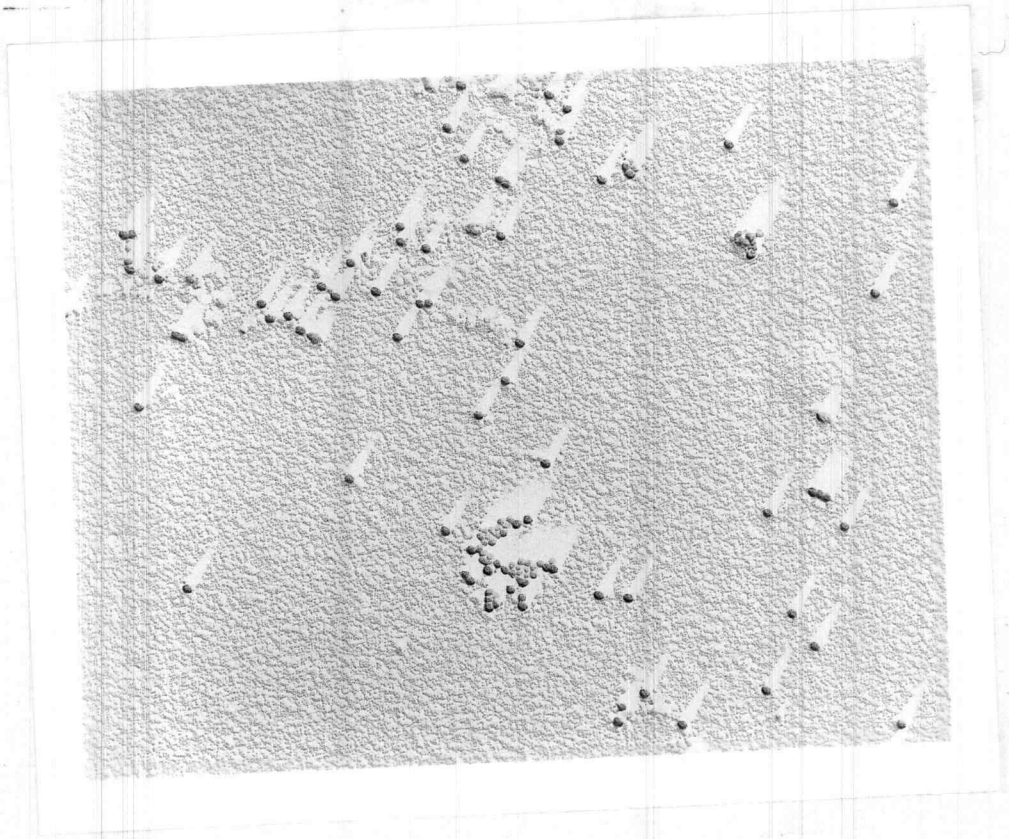

Figure 4. Shadow-casting of AEV with chrome (50,000 X).

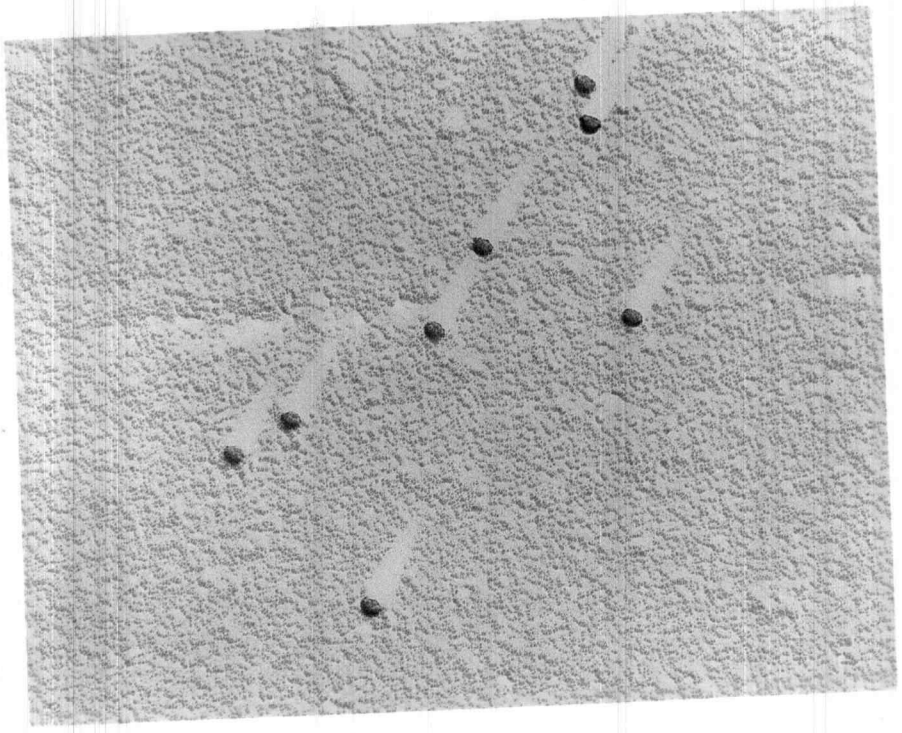

Figure 5. Shadow-casting of AEV with chrome (111,000 X).

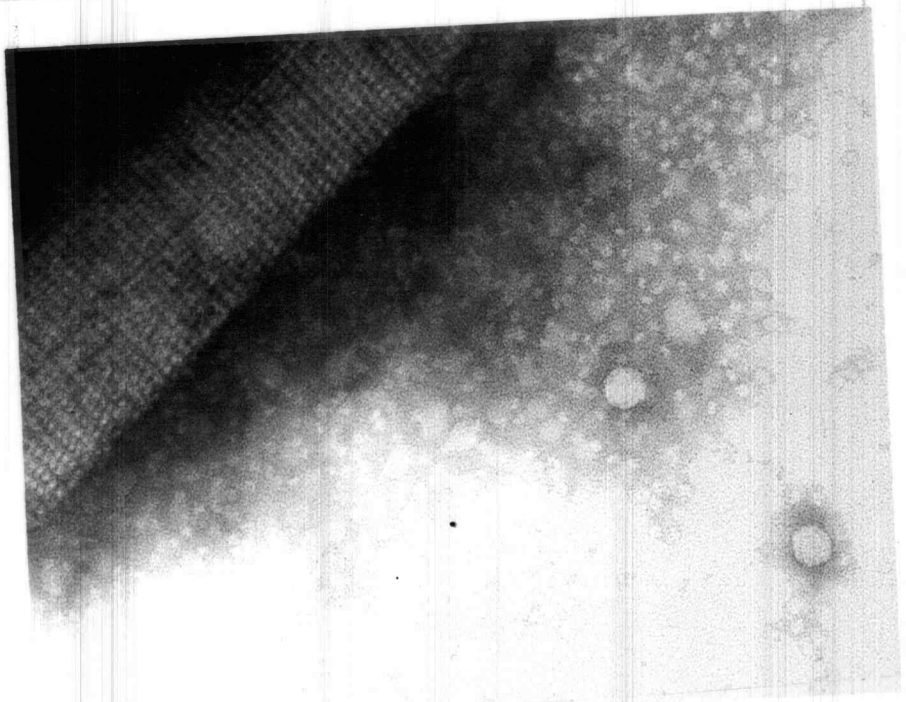

Figure 6. Negative stain of bovine catalase crystal and AEV (217,000 X).

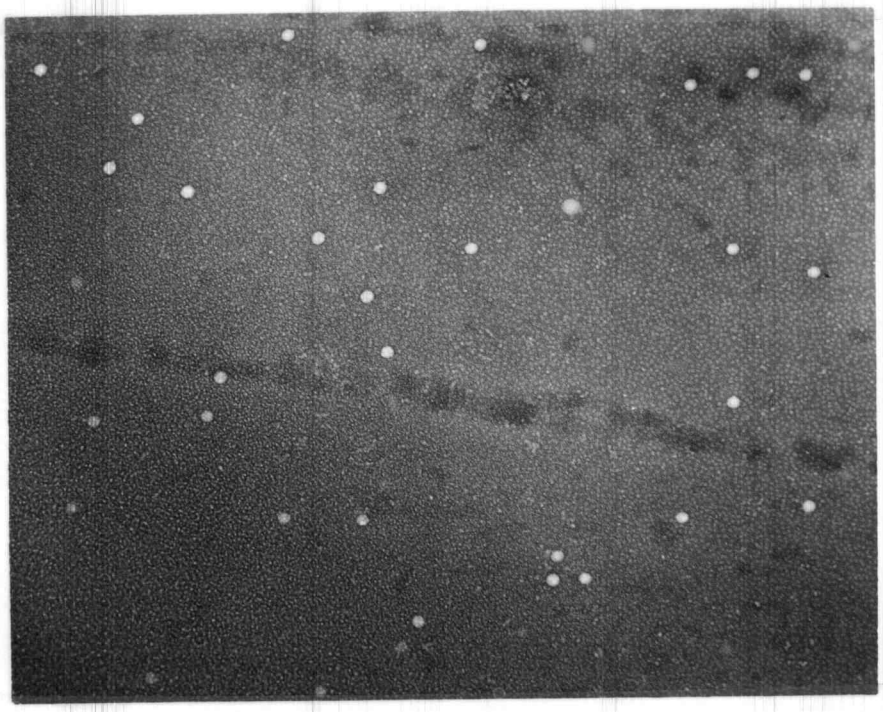

Figure 7. Negative stain of AEV with sodium phosphotungstate (67,300 X).

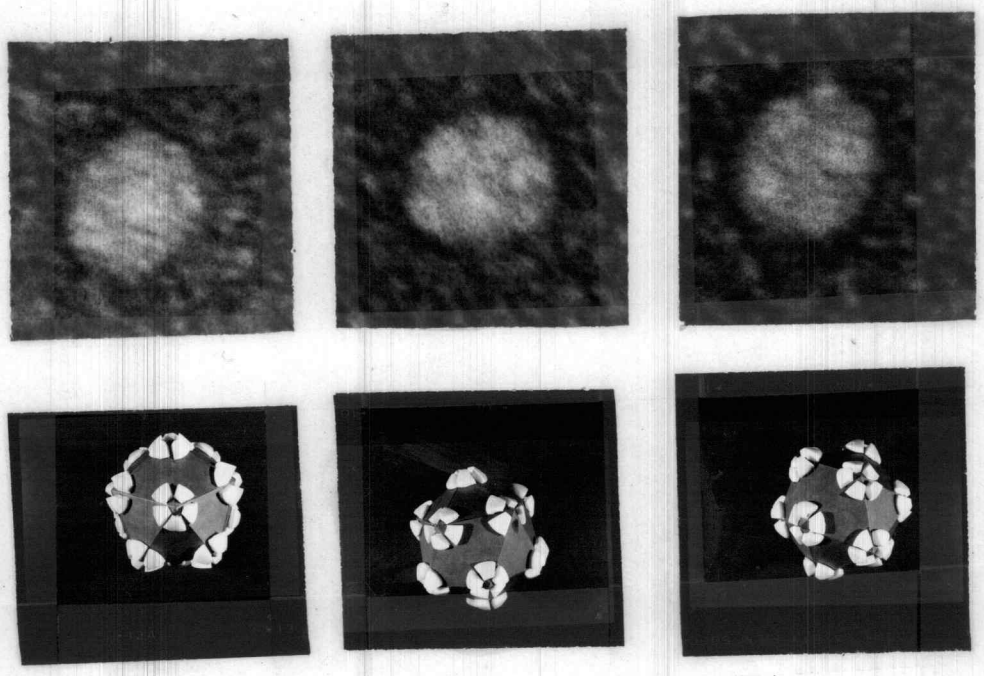

Figure 8. Comparison of virus particles with an icosahedral model with 12 capsomers located at the vertices (7,700,000 X).

particles (Figures 8a-8f). Some capsomers could be resolved into five structural units. Models were constructed which were icosahedral in shape with 12 capsomers located at the vertices. The models were oriented in a similar manner to the particles in the micrographs and photographed for comparison with the virus particles.

Sensitivity to trypsin

Purified virus samples were treated with four concentrations (1, 0.5, 0.250, and 0.125%) of trypsin. The treatment did not produce a decrease in infectivity from that of the control. All values fell within one log difference of the control.

Acid sensitivity

The virus was subjected to diluents of pH 3.0 and 7.1 at room temperature for one hour. The EID_{50} of the sample in pH 3.0 diluent was $10^{-4.9}$/0.1 ml. The control sample had an infective titer of $10^{-4.3}$/0.1 ml. It was concluded that AEV was stable to acid pH under these experimental conditions.

Sensitivity to chloroform

The aqueous sample of AEV which was saturated with chloroform for at least ten minutes did not show any decrease in infectivity in comparison to the control. The sample treated with chloroform had an EID_{50} of $10^{-4.7}$/0.1 ml as compared to $10^{-4.2}$/0.1 ml for the control. These results indicate the strain of AEV used in the study was not

susceptible to inactivation by this lipid solvent.

Heat inactivation and stabilization by 1 M $MgCl_2$

An AEV sample which was incubated at 56 C for one hour showed a decrease in titer of 1.2 $\log_{10}$ in comparison with a similar sample which was kept at 4 C for the same period of time. The presence of 1 M $MgCl_2$ stabilized the virus against inactivation by heat, and full infectivity of the AEV sample was retained. The EID_{50} of the sample kept at 4 C was $10^{-4.7}$/0.1 ml as compared with a titer of $10^{-3.5}$/0.1 ml for the sample incubated at 56 C. The virus sample containing 1 M $MgCl_2$ had an EID_{50} of $10^{-4.5}$/0.1 ml.

Nucleic acid type

Several methods were used in attempts to determine whether AEV has a RNA or DNA genome. Treatment of intact virions with RNase and DNase did not cause a demonstrable decrease in infectivity of the virus. The control had an EID_{50} of $10^{-4.6}$/0.1 ml while samples treated with DNase and RNase had EID_{50} titers of $10^{-5.4}$/0.1 ml and $10^{-5.3}$/0.1 ml respectively. The extraction of infectious nucleic acid from the purified virus could not be demonstrated in embryonating eggs. Inhibitors of nucleases could not be utilized in the extraction since they would inhibit the action of the enzymes used in the experiment. Virus samples were air-dried on coverslips and subjected to RNase and DNase before staining with acridine orange. This procedure was used to study poliovirus and tobacco mosaic virus by Mayor and Diwan (1961)

but it was unsuccessful in this experiment. All slides exhibited a bright green fluorescence which was not removed by treatment with either nuclease. The product of a phenol extraction was used in an experiment which attempted to determine the type of nucleic acid by the diphenylamine colorimetric method and the orcinol colorimetric method. Negative results were obtained by both methods. Finally, it was attempted to label propagating virus with radioactive pyrimidine precursors in chicken embryo fibroblast cell cultures. Labelling for 24 hours with ^{3}H-uridine failed to demonstrate an activity peak which corresponded to the determined location of the virus in the gradient, and which was also absent from the control (mock-infected) cell culture.

DISCUSSION

The bouyant density of AEV in cesium chloride was determined by assaying the infectivity of fractions collected following isopycnic gradient centrifugation. Repeated experiments demonstrated that the major peak of infectivity corresponded to densities of 1.31 to 1.32 g/ml (Figures 1a and 2). Minor peaks were observed at densities of 1.40 and 1.36 g/ml. Sonication and filtration through cellulose filters of 0.22μm retention size failed to remove the minor peaks. The reproducibility of the smaller peaks argues against the possibility that they represent nonspecific association of the virus particle with host cell components.

Butterfield et al. (1969) reported a density of 1.33 g/ml for AEV. When they layered the brain homogenate onto a solution of CsCl of density 1.2 g/ml and performed an equilibrium centrifugation, they detected infectious virus throughout the gradient. This could be explained by the association of the viral particles with less dense lipids and lipoproteins. In the present study, a large amount of lipids and nonviral proteins were removed by the treatment with PEG and fluorocarbon before the sample was layered onto the CsCl solution. As the results indicate (Figures 1a and 2), the separation of the viral peaks was clear and reproducible. In the present study, density values were determined by both weight measurement and refractive indices of the fractions. The values obtained by the two methods coincided well (Figure 1b) and error in either technique was less than 4%. Density

values can vary according to the experimental conditions of the determination, and differences in values have been reported between serotypes of the same type of virus. For example, Newman et al. (1973) noted that the bouyant densities of foot-and-mouth disease virus (FMDV) ranged from 1.42 to 1.44 g/ml under standard sedimentation conditions. Also, the value of 1.33 g/ml was determined on a non-purified sample.

Since AEV shares many similar characteristics with both picornaviruses and parvoviruses, many of the chemical and physical properties which were determined for AEV in this study will be compared with corresponding reports in the literature for the two genera. The bouyant densities of picornaviruses which have been reported in the literature are as follows: poliovirus, 1.34 g/ml (Mattern, 1962; Schaffer and Frommhagen, 1965); bovine enterovirus, 1.34 g/ml (Martin et al., 1970); vesicular exanthema virus, 1.36 to 1.38 g/ml (Oglesby, 1965; Wawrzkiewicz et al., 1968); feline picornavirus, 1.37 to 1.39 g/ml (Studdert et al., 1970); human rhinovirus, 1.38 to 1.41 g/ml (Chapple and Harris, 1966; Dans et al., 1966; McGregor et al., 1966; Gerin et al., 1968); FMDV, 1.43 to 1.51 g/ml (Trautman and Breese, 1962; Liebermann and Gralheer, 1968). Even though most picornaviruses exhibit similar size and composition, there exists a wide range of bouyant densities in CsCl within the family (Rowlands et al., 1971). Three possibilities were suggested which could account for the range in densities. The more dense viruses could contain more RNA in relation to the amount of protein. Secondly, the more dense viruses could have a

greater degree of reactivity of the RNA with the cesium ions because of greater accessibility. Also, the different viruses could have different degrees of hydration. The values of percentage RNA and molecular weights of the respective picornaviruses presented by Newman et al. (1973) would argue against the first possibility. Both sets of values fall within a narrow range. Rowlands et al. (1971) presented some interesting results concerning the effects on density of picornaviruses of duration of centrifugation, pH, prolonged storage, and anions of cesium salts. The acid-stable enteroviruses were unaffected by these factors. Density values of acid-labile FMDV increased with the duration of the centrifugation and also with increased pH values. The density of FMDV increased from 1.43 to 1.46 g/ml after storage at 2 C for 25 weeks. All groups, except the enteroviruses, showed a decrease in density when cesium sulfate was used to form the gradient instead of cesium chloride.

Kilham rat virus (RV), type species of the genus Parvovirus, and H-1 virus, which was isolated from the transplantable human tumor HEp-1, separate into two main bands during density gradient centrifugation in CsCl. The density of the light band is 1.30 to 1.32 g/ml in CsCl (Jamison and Mayor, 1965). The heavy band has a density of 1.40 g/ml. RV bands in potassium tartrate gradients at densities around 1.31 to 1.32 g/ml (Breese et al., 1964). An additional peak in CsCl has been reported at 1.38 g/ml (Robinson and Hetrick, 1969), which was shown by electron microscopy to be composed of full and empty capsids. Full infectivity is associated with the heavy band, while

the light band has low infectivity. Minute virus of mice (MVM) has been reported to band in CsCl at densities of 1.41 to 1.43, 1.38, and 1.35 g/ml (Crawford, 1969).

The examination of purified AEV with electron microscopy using negative stain revealed virus particles which were icosahedral in shape, lacked an envelope, and had a mean diameter of 26 $\pm$ 3 nm. Krauss and Ueberschar (1966) reported a size of 23.3 nm with a standard deviation of 2 nm for AEV. The difference between these values can not be considered significant in light of the variations between values which have been reported for many of the small (less than 30 nm) viruses. The estimated size of poliovirus has ranged from 20 to 24 nm (Mayor, 1964) to 27.5 to 30 nm (Agrawal, 1966). The diameters reported for the hampster-osteolytic viruses have ranged from 20 to 29 nm (Toolan et al., 1964). Some of the variations might be due to methods of sample preparation which could result in a distortion or flattening of the viral particles. Calibration of the electron microscope could be another source or error. Bovine catalase crystals were added in this present study to the virus sample to serve as an internal standard for measurement. Wrigley (1969) notes that the orientation of an icosahedral virus must be known for an accurate measurement, since 'face-to-face' and 'corner-to-corner' diameters differ by 11.5%. A mean diameter of 26 $\pm$ 3 nm falls within the accepted ranges for both picornaviruses and parvoviruses.

Crick and Watson (1956) are accredited with the concept that all small viruses are constructed of identical protein subunits which

are arranged in a regular fashion to provide a protective shell for the nucleic acid. The advantage of icosahedral symmetry is that it allows the use of the greatest number (60) of identical asymmetric units to build a spherical form (Casper and Klug, 1962). The efficiency of icosahedral symmetry is clear when the ratio of surface area to volume enclosed is compared for tetrahedrons (1:1), cubes (1:2.4), octahedrons (1:2), dodecahedrons (1:5.4), and icosahedrons (1:3.6) (Mayor and Jamison, 1966). Both forms with icosahedral symmetry (icosahedron and dodecahedron) have the lowest ratios.

The small size of picornaviruses and parvoviruses has made the determination of the exact number of capsomers and their arrangement a difficult task. This information is still a source of controversy surrounding many of the small viruses. Analysis of the fine structure of several parvoviruses have suggested that the capsid is constructed of 32 subunits (Siegl, 1976). However, the most recent model, which is based on the relative amount of viral polypeptides within a H-1 virus particle, has reconsidered an icosahedron composed of only 12 pentamers (Kongsvik et al., 1974). The bacterial virus, ϕ X 174, has been shown to possess only 12 capsomers. To my knowledge, no picornavirus which infects vertebrates has been shown to contain 12 capsomers.

Examinations of negatively stained AEV suggest that the capsid of AEV is constructed of 12 morphological units. This is in agreement with the finding of Krauss and Ueberschar (1966). It is further suggested that the capsomers or morphological units, are, in effect, 12 pentamers located at the vertices of an icosahedron. The resolution

of some capsomers allows the detection of the separate subunits.

Casper and Klug (1962) describe several numerical relationships which can be used to describe the structural characteristics of an icosahedron. The relationship between the number of morphological units and the triangulation number (the number of smaller identical triangles into which an equilateral triangular face of an icosahedron can be divided) is : $M = 10\ T + 2$. A virus which has 12 morphological units would have a triangulation number of one. The relationship between the triangulation number and the class of icosahedral deltahedra (P) is : $T = P\ f^2$ where f is any integer. Therefore, a virus with a triangulation number of one necessarily belongs to the class $P = 1$. The number of structural units is described by the equation : $S = 60\ T$. It follows that a capsid described by a triangulation number of one has 60 structural units. It is suggested that AEV can be described by a triangulation number of one, as belonging to the class $P = 1$, and as having 60 structural units in the capsid.

The sedimentation coefficient ($S_{20,w}$) of AEV was determined to be 148 ± 3 S. This value correlates with the sedimentation coefficients of small RNA viruses infecting vertebrates which range from 146 to 160 S (Brown and Hull, 1973). The RNA bacteriophages have reported values between 71 and 84 S. Small RNA viruses infecting plants have reported $S_{20,w}$ values ranging from 83 to 132 S. The sedimentation coefficients for parvoviruses have reported values of 105 to 110 S (Siegl, 1976). The bacteriophage, ϕX 174, has a $S_{20,w}$ of 112, which is similar to those reported for parvoviruses (Crawford et al., 1969).

A sedimentation velocity run will yield information about the possible heterogeneity of a virus preparation (Kaper, 1968). Only one boundary was observed in the moving boundary experiments with AEV. While this is not definitive proof of the homogeneity of the sample, it does give credence to the relative purity of the sample.

Avian encephalomyelitis virus was shown to be stable at pH 3.0 for one hour. This is in agreement with the results of Butterfield et al. (1969). Stability at pH 3.0 is used to distinguish between the enteroviruses and rhinoviruses. The infectivity of the enteroviruses is unaffected by this treatment, whereas the infectivity of the rhinoviruses is considerably reduced (Tyrrel and Chanock, 1963). All members of the genus Parvovirus have been shown to be stable at pH 3.0 (Siegl, 1976). Since AEV has been shown to initiate infection via the intestinal tract (Calnek et al., 1960), it is not surprising that the virus is stable in acid pH.

The infectivity of AEV was not decreased after treatment with chloroform. Inactivation of viruses containing essential lipids by chloroform and ether has been widely used in the classification of viruses. There appears to be a correlation between the presence of an envelope and sensitivity to lipid solvents. Since an envelope was not detected by electron microscopy, and an organic solvent was successfully used by Matsumoto and Murphy (1977) in the partial purification of AEV, it would have been surprising should the virus proved to be sensitive to treatment with chloroform. Both picornaviruses and parvoviruses are resistant to inactivation by chloroform (Siegl, 1976;

Philipson, 1967).

A drop of 1.2 $\log_{10}$ in the infective titer of AEV was demonstrated after heating at 56 C for one hour. The presence of 1 M $MgCl_2$ stabilized the virus against inactivation by heat. The presence of 1 M $MgCl_2$ stabilizes enteroviruses and reoviruses to heating at 50 C for one hour, and no loss of infectivity is observed (Wallis and Melnick, 1962). Adenoviruses, papovaviruses, herpesviruses, myxoviruses, arboviruses, and poxviruses are inactivated by heat more readily in the presence of 1 M $MgCl_2$ (Wallis et al., 1962). However, the taxonomic attributes of inactivation by heat and stabilization by cations are of limited value (Wilner, 1969).

Demonstration of the type of nucleic acid will most likely be determined in a definitive manner in a cell culture system which allows unquestionable propagation of the virus. Such a system would allow the investigator to utilize isotope-labelled pyrimidine precursors. Demonstration of the incorporation of either labelled uridine or labelled thymidine into the viral genome would settle the question in a satisfactory manner. Once a system for propagating the virus in cell culture was devised, the use of deoxyuridine analogues could also play a useful role, although the use of base analogues is less favored since it is now known that some RNA viruses require the synthesis of DNA shortly after infection.

The use of embryos as the only adequate source of AEV and as the only system for assay of the virus presents difficulties in attempts to determine the nature of the viral genome. Although the virus can be

propagated in high titers (10^6 to 10^7 EID_{50}) in the embryo, the purification steps necessary to eliminate the host components from the preparation also reduce the yield of purified virus. Physical methods of nucleic acid determination such as orcinol and diphenylamine colorimetric methods require concentrations of nucleic acid which were not achieved in this study. The same holds true for acridine orange staining. Mayor and Diwan (1961) calculated that the equivalent of 50 closely packed poliovirus particles was necessary for the visualization of the fluorescing dye in the fluorescence microscope.

Most viruses are resistant, unless denatured, to treatment by proteolytic enzymes and nucleases (Knight, 1975). For this reason, it is not surprising that purified AEV was unaffected by treatment with RNase and DNase. The reduction in titer, 0.7 $\log_{10}$, reported by Butterfield et al. (1969) after treatment with RNase has to be critically appraised. The magnitude of the decrease is such that it could reflect variation within the assay system. It is also very difficult to demonstrate infectious nucleic acid in an embryo because of the presence of nucleases.

Chick embryo fibroblast cell cultures were prepared for use in labelled precursor studies. Attempts to show the incorporation of ^3H-uridine into the virus were unsuccessful. It is not known whether this was a result of a failure of the virus to propagate in sufficient numbers to allow detection of the activity above mock-infected controls or whether the virus does not contain RNA.

Classification of AEV as a picornavirus (Butterfield et al.,

1969) was premature since the nature of the viral nucleic acid has not been shown in a definitive manner. The use of density is not a valid means for differentiating picornaviruses and parvoviruses. The enteroviruses and parvoviruses share many characteristics such as resistance to inactivation by heat, resistance to lipid solvents, small diameters, stability in acid pH, and icosahedral symmetry. The sedimentation coefficients of parvoviruses are lower than those of enteroviruses, and the density of infectious particles is higher for parvoviruses, but the only definitive criterion for differentiating the two remains the nature of the nucleic acid. Until the nucleic acid type is demonstrated for AEV, the virus can not be classified in either the Picornavirus or Parvovirus genus.

The present study, however, demonstrated physico-chemical as well as biological characteristics of purified AEV. The information concerning the purification and concentration of AEV is valuable to the efforts directed towards the development of a convenient diagnostic test for AE infection.

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