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Title: MORPHOLOGY AND NUCLEIC ACID CHARACTERIZATION OF AVIAN ENCEPHALOMYELITIS VIRUS

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The purpose of the present study was to examine the surface structure of avian encephalomyelitis virus (AEV) and to characterize the viral nucleic acid. The virus was purified by methods previously described and further by isopycnic centrifugation in CsCl. This purified virus had a buoyant density of 1.30 to 1.32. Electron microscopy showed the virions to be hexagonal with diameters varying from 24 to 32 nm depending on the preparation method. Icosahedral, 5:3:2, symmetry was demonstrated with the Markham rotation technique. From rotations on uranyl acetate stained particles, a 32 or 42 capsomer model was proposed. Virus propagated in whole chick embryo cell cultures resulted in low infectivity titers that persisted for up to 16 days post inoculation. An RNA genome for AEV was suggested since 5'-bromodeoxyuridine (BUDR) had no effect on viral growth in these cultures. Additional evidence for an RNA genome was successful labelling of virus with tritiated uridine but not with tritiated thymidine in cell culture and RNAse sensitivity of isolated viral nucleic acid.

Direct chemical analysis of purified virus indicated a virion RNA content of 27.1%. The viral nucleic acid had a sedimentation coefficient of 33.6 S and molecular weight values of 2.3 and 2.4 x 10^6 daltons by polyacrylamide gel electrophoresis and sedimentation studies, respectively. The virion molecular weight was estimated to be between 7.6 and 8.5 x 10^6 daltons. From ultraviolet absorbance studies, an extinction coefficient ($E^{1\%}_{260}$) of 74 was determined for the virion. Based on these results, AEV was classified in the family Picornaviridae, genus Enterovirus.

Morphology and Nucleic Acid Characterization of Avian Encephalomyelitis Virus

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INTRODUCTION

Avian encephalomyelitis virus (AEV) has not been well characterized biochemically or morphologically. Nucleic acid type, the definitive criterion for classifying the virus, is unknown. The purpose of the present study was to examine the morphology of the virus and determine the nucleic acid type.

Literature Review

The Infection

Avian encephalomyelitis (AE) is an infectious disease of poultry caused by a virus (Luginbuhl and Helmboldt, 1978). Naturally susceptible hosts include chickens, turkeys, and the coturnix quail (Van der Heide, 1975). The clinical disease in chickens occurs in ages between one and four weeks and causes significant economical losses. The disease in young birds is characterized by neurologic signs consisting of ataxia, incoordination, paralysis or tremors. The affected birds seldom recover and mortality can be as high as 50%. In contrast, the infection in adult birds (older than six weeks) is asymptomatic except for a transient drop in egg production in laying hens. In many instances, seroconversion is the only means of detecting infection in older birds (Luginbuhl and Helmboldt, 1978).

Characteristic histological lesions appear in the central nervous system (CNS) of the infected birds. These are of a disseminated non-purulent encephalomyelitis consisting of degeneration of neuonal cells, ganglionitis of the dorsal root ganglia, perivascular cuffing and a general hyperplasia of the lymphatic tissues (Luginbuhl and Helmboldt, 1978). Other lesions, in other parts of the host, are either rare or nonpathogenic.

Natural transmission of the disease to chicks was shown to result from vertical egg-borne infection (Taylor et al., 1955) and from direct or indirect contact (Calnek et al., 1960). AE was suggested to be an enteric infection with the virus eliminated in the feces in sufficient quantities to infect susceptible chicks by the oral route. In fact, oral administration of AEV induces a syndrome indistinguishable from that seen in naturally occurring outbreaks (Calnek et al., 1961).

Immunization against AE was successful with both live and killed vaccines. Sufficient amounts of maternal antibodies can be induced in dams to protect the progeny through the susceptible period. Vaccination effort, therefore, was limited to breeders. Calnek et al. (1961) successfully vaccinated by oral administration of a live field strain. In addition to the live vaccines, a beta-propiolactone inactivated virus preparation offers excellent protection for months (Butterfield, 1975). This vaccine was recommended for birds close to or in production because immunization with a live virus could result in infected progeny. A lyopholized vaccine also was used with success (Polywaczyk et al., 1972). The protective immunity to AEV infection was humoral in nature since, regardless of age, infection was not altered in

thymectomized birds but drastically enhanced by bursectomy (Cheville, 1970).

For detecting infection and evaluating immune status, serological identification was performed with serum neutralization tests (Sumner et al., 1957) using an embryo-adapted virus strain and AE susceptible embryonating eggs. In addition, an embryo susceptibility test was used to determine infection in breeding flocks (Sumner et al., 1957). Direct fluorescent antibody (FA) testing was successful in demonstration viral antigens in AEV infected birds (Braune and Gentry, 1971a, b; Sato et al., 1971). Indirect FA (Choi and Miura, 1972) and FA blocking tests (Davis and Lukert, 1971) were reported but are seldom used because of their unreliability. Matsumoto (1978) has developed, using purified antigen, a gel diffusion test to detect antibody against AEV.

Based on the serum neutralization test, all reported AE virus isolants and vaccine strains are of one serological type (Van der Heide, 1975). On the other hand, the biological properties of these various isolates are quite different. The natural or field strains are all enterotropic, infecting young chicks by the oral route and shed in the feces (Luginbuhl and Helmboldt, 1978). The neuotropic properties, however, vary among the AE isolates. Field isolates usually are nonpathogenic for embryos unless they are successively passed in the embryo. These embryo-adapted strains become neurotropic causing the neurological lesions in embryos. Parenteral injection of any AE strain causes typical lesions and symptoms in susceptible chicks.

AEV was found to multiply in a number of tissues in vivo. Both fluorescent antibody staining and titration of tissues has been used to detect growth. Burke et al. (1965) demonstrated that in embryos inoculated with an embyro-adapted strain of virus, the greatest multiplication occurred in the brain and viscera. In birds of all ages, growth of the virus was shown to occur in the brain (Ikeda et al., 1976a,b,c; Miyamae, 1974; Braune and Gentry, 1971). In addition to the CNS, one of the major sites of replication is the pancreas (Miyamae, 1974). The viral antigen tends to persist in this organ for several weeks. Therefore, replication in the pancreas is considered to have an important role in the immunization of chickens against the virus. Miyamae (1978) in fact showed that serial pancreatic passage of the virus decreased invasiveness to the CNS yet the virus retained pancreotropism and the ability to stimulate high titers of neutralizing antibody. The above investigators also demonstrated viral antigen in most of the parenchymal organs and in the muscles of the leg.

In contrast to the growth in vivo, virus replication is considerably lower in cell culture. Because host tissues would most likely support the growth of AEV, investigators propagated the virus in a number of primary embryo and chicken cell cultures including whole embryo cells (fibroblasts)(Mancini and Yates, 1968b), embryo kidney cells (Mancini and Yates, 1968a), embryo brain cells (Mancini and Yates, 1967; Sato et al., 1971; Komada et al., 1974), and chicken pancreatic cells (Kodama et al., 1975). The highest titers were observed in the brain cell cultures. The virus was not demonstrated

to replicate in any established cell lines although numerous attempts were made (Abe, 1968). Growth in monkey kidney cells was reported by Halpin (1966), but was not confirmed.

The Virus

The buoyant density in CsCl was reported to be 1.33 g/ml (Butterfield et al., 1969) and 1.31 to 1.32 (Gosting, 1977). A sedimentation coefficient of 148 ± 3 S was determined by the latter investigator. The size of the virion was estimated to be 20 to 30 nm by gradicol filtration (Olitsky and Bauer, 1939) and again by filtration a range of 17 to 25 was reported (Butterfield et al., 1969). Measurements by electron microscopy gave values of 23.3 ± 2 nm (Krauss and Ueberschar, 1966) and 26 ± 3 nm (Gosting, 1977). These studies indicated that the virus had an icosahedral shape, lacks an envelope and possibly had a 12 capsomer structure. The virus was determined to be stable in acid (Gosting, 1977; Butterfield et al., 1969) and chloroform (Gosting, 1977; Butterfield et al., 1969; Buelow, 1964; Krauss and Ueberschar, 1966). Incubation with trypsin had no effect on viral infectivity (Gosting, 1977; Buelow, 1964). AEV was liable to heat at 56 C for one hour but was stabilized to these conditions by divalent cation (Gosting, 1977; Butterfield et al., 1969; Buelow, 1964). Butterfield et al. (1969) determined that the virus was slightly affected by ribonuclease. A $10^{0.7}$ decrease in the median-embryo-infective dose was observed. On the basis of this result, AEV was classified as an Enterovirus. Gosting (1977) on the other hand, showed no sensitivity of purified AEV to ribonuclease or deoxyribonuclease.

Classification of Viruses

The criterion for the classification of viruses has changed since the early proposal of Holmes (1948) that based classification on the disease and host responses rather than the properties of the virus. Bawden (1941) suggested that viruses be classified on the basis of the particle. At the International Microbiological Congress of 1950 (Andrewes, 1952), Bawden's approach was partially adopted and eight criteria were formulated for the classification of viruses.

1. Morphology and method of reproduction
2. Chemical properties and physical properties
3. Immunological properties
4. Susceptibility to chemical and physical agents
5. Natural methods of transmission
6. Host, tissue and cell tropisms
7. Pathology and inclusion body formation
8. Symptomatology

Andrewes *et al*. (1961) considered that the first two criteria were most important because they believed that the major virus groups could be defined by a few fundamental characteristics of the mature virion with less emphasis on the "vegetative" phase. Therefore, a method of classification was proposed based on four fundamental characteristics of the virion;

1. The nature of the genetic material
2. The symmetry of the capsid
3. Enveloped vs nonenveloped nucleocapsid

4. The number of capsomers (for cubic viruses) (Lwoff and Tournier, 1966). Groups of viruses defined by these characteristics were considered families. A hierarchy of 29 additional characteristics were enumerated for further grouping. Lwoff and Tournier (1971) indicated that this classification scheme was opposed. The main argument was directed towards the arbitrary nature of the proposed hierarchy.

In 1966, Gibbs et al. proposed the use of four pairs of characteristics for grouping viruses, expressed in the form of a cyrptogram. The first term described the type of nucleic acid (R=RNA, D=DNA) and the strandedness (1,2 = single, double stranded). The second term described the molecular weight of the nucleic acid (in millions) and the percentage in the virion. If the virion had a segmented genome, it was proposed (Wildy, 1971) that the symbol followed by the total genome weight be used. The third term gave the outline of the virion, outline of the nucleocapsid (S = spherical, E = elongated, X = complex). The fourth term described the kind of host infected (V = vertibrate, I = invertibrate), the kind of vector (0 = none, Di = diptera, Ac = tick, mite). The crypogram was, in many ways, a culmination of the previous classification methods.

Bellett (1967) proposed classifying viruses on the basis of the molecular weight of the nucleic acid and the percentage of guanine plus cytosine. A similar proposal, Lanni's system, was based on the sequence of the codons (Lwoff and Tournier, 1971). Both methods have merit because the genetic material specifies the characteristics of the virus. However, the former proposal would classify a reovirus

into the same basic group as the double stranded DNA viruses. The later proposal was viewed as inapplicable because sequencing of nucleic acid, even in view of recent advancements, was not feasible for general viral classification.

The International Committee on the Nomenclature of Viruses (ICNV) (Tenth International Congress for Microbiology, 1970) proposed a dual system of nomenclature consisting, on the one hand, of generic names ending in 'virus' and the eight digit crypotgram to describe each virus. As indicated by Luria et al. (1978) this system appears not to have found favor with virologists.

Only two families were established by the ICNV in 1970 (Wildy, 1971): Papovaviridae and Picornaviridae. Subsequently, the family Togaviridae was defined (Fenner, 1974). Most accepted "groups" of viruses were given generic names.

The second report of the International Committee on Taxonomy of Viruses (ICTV)(Fenner, 1976) stated the current official determinations on viral taxonomy. Melnick (1978) schematically outlined the criterion for genus classification in the following order:

1. Capsid symmetry
2. Naked or enveloped virion
3. Site of capsid assembly (DNA viruses)
4. Site of nucleocapsid envelopment
5. Reaction to lipid solvent
6. Number of capsomers (or diameter of helix)
7. Diameter of virion
8. Molecular weight of nucleic acid

All major virus groups were given family names even if they contained only one genus.

The Fourth International Congress for Virology (September, 1978) was held to discuss recommendations of ICTV Study Groups. The study group on the family Picornaviridae set forth several recommendations for major change. The family should at present comprise four genera: Enterovirus, Cardiovirus, Rhinovirus and Aphthovirus. Aphthovirus is the new genus for foot-and-mouth disease virus. In addition, it was recommended to separate the Calicivirus into a new family.

MATERIALS AND METHODS

Virus

A strain of embryo-adapted AEV (Sumner et al., 1957) was used as the test virus for all the studies. Virus was propagated in embryos originated from White Leghorn breeders (Matsumoto et al., 1978). Serum neutralization tests (Calnek and Jehnich, 1959) showed these birds to be negative for antibodies against AEV. Six-day-old embryos were inoculated with 0.1 ml stock virus via the yolk sac. The virus was diluted; each embryo received 100 median-embryo-infective dose (EID_{50}). Hanks balanced salt solution containing 0.5% lactalbumin hydrolysate (HLH)(GIBCO), 100 I.U./ml penicillin and 100µg/ml streptomycin was used for all virus dilutions. Stock virus consisted of a 10% suspension of brain from infected embryos.

Infectivity Assay

Virus titers were determined by yolk sac inoculation of 0.1ml of the appropriate sample dilution in six-day-old embryos. Twelve days post inoculation, the embryos were examined for typical AE lesions as described by Luginbuhl and Helmboldt (1978). Median-embryo-infective dose (EID_{50}) was calculated by the method of Kärber (1931).

Purification of AEV

Virus was purified according to the methods of Matsumoto and Murphy (1977) and Matsumoto et al. (1978). Briefly, embryos,

inoculated as above were incubated for 10 to 12 days and those showing typical lesions, were homogenized in a 50% suspension in HLH with a waring blender. After three cycles of freeze-thaw, the suspension was centrifuged at 1000 x g for 15 minutes to remove large debris. The virus was precipitated from the supernatant by the addition of polyethylene glycol (PEG) 6000 (Fisher Scientific Co.) to a final concentration of 4% (w/v). Precipitation was performed at 4 C for 90 minutes while stirring. The precipitate was collected by pelleting at 6,000 x g for 30 minutes in a Sorval GSA rotor. The pellet was resuspended in tris-HCl buffered saline (TBS) that contained 0.125 M Tris, 0.15M NaCl, pH 7.6, to the original volume of homogenate. The virus was separated from lipid and protein contamination by extraction with an equal volume of trichlorotrifluoroethane (TTE; Freon, DuPont de Nemours). After centrifugation at 6,000 x g for 30 minutes, the aqueous phase that contained the virus was collected. At this point the preparation was either ultrafiltered in an Amicon model 402 ultrafiltration cell with a XM membrane, and/or directly centrifuged at 73,000 x g for 150 minutes in a type 30 rotor (Beckman). The resulting virus pellet was resuspended in three to six ml of TBS and clarified by centrifugation at 3,000 x g for 30 minutes. The supernatant was removed and the sedimented materials were resuspended in three ml of TBS and again centrifuged. The supernatants were pooled.

For further purification, the virus was centrifuged in a non-linear CsCl gradient. The gradient was prepared by layering one ml of 1.29 density CsCl on to one ml of 1.34 density CsCl in nitrocellulose tubes of 5 ml capacity. Three ml of the crude virus

concentrate was layered carefully on top of the gradient, and the tube was centrifuged in an SW 50.1 rotor at 192,000 x g for 120 minutes. The virus band, at the interface of the two CsCl layers was collected from the bottom of the tube using a Beckman Universal fraction collector. The volume of this fraction, designated Fraction 2, was 0.4ml. The CsCl was removed by dialysis against TBS. Fraction 2 was used for electron microscopy.

Further purification was obtained by isopycnic centrifugation in CsCl. Fraction 2 was mixed with 4.5ml of CsCl of 1.34 density and centrifuged at 192,000 x g for 18 hours. The virus band was collected with thin tubing connected to a syringe. In one experiment the gradient was fractionated and the infectivity of each fraction was determined for comparison to densities. Density of each fraction was determined by weighing a 250 µl sample in a Lang-Levy pipette. Fraction 2 was mixed with an equal volume of a nuclease solution containing 90 units/ml ribonuclease A (RNAse, 4590 units/mg, Worthington Biochemical Co.) and 30 units/ml deoxyribonuclease I (DNAse, 2000 units/mg, Worthington) in 0.01 M $MgCl_2$. The mixture was incubated at 37 C for 30 minutes and centrifuged in the isopycnic gradient.

AEV also was purified by chloroform extraction of infected brain material (Krauss and Ueberschar, 1966). For comparative purposes, AEV prepared by this method was examined by electron microscopy.

Electron Microscopy

All virus preparations, unless otherwise stated, were negatively stained by mixing with an equal volume of 2% phosphotungstic acid (PTA)

at pH 6.5-7.0. Preparations were applied to carbon-coated formvar films (0.25% in ethylene dichloride) supported on 400 mesh copper grids with a glass nebulizer (Model No. 40, DeVilbiss Co.). Grids were examined in a Phillips EM300 electron microscope at 80 or 60 kv. Virions were measured on the original glass image plates using bovine catalase crystals as an internal standard of measurement (Wrigley, 1968).

Virus samples were treated with various agents prior to staining with PTA. AEV, in TBS, was diluted with an equal volume of the nasal decongestant "Afrin" (Schering Corp.) and kept at room temperature for 48 hours. Other samples were suspended in 0.05 M tris-HCl buffer (pH 7.4) containing 1.0 M urea, 0.04% Triton X-100, 1.25% trypsin (Difco) or 0.1M phosphate buffer at pH 4.5 or 0.05 M Tris-HCl at pH 10.5. Samples treated with these agents were centrifuged at 192,000 x g for 120 minutes at 20 C, and the pellets were resuspended in distilled water.

In addition to the PTA staining, the following methods were used to examine surface detail. Samples were positively stained with uranyl acetate (UA) by floating a virus coated grid on a drop of 1% UA (pH 4.4) and of Reynold's lead citrate successively (Reynolds, 1963). In another experiment, the virus coated grid was fixed on a drop of 1% UA for five minutes.

A replica of AE virus was prepared by the method of Hall (1956) modified as follows. Purified virus was applied onto the surface of freshly cleaved mica and then shadowed with platinum in a vacuum evaporator at 0.45 degree angle. For additional support, the sample

was shadowed with carbon. This replica was separated from the mica by floatation on water and mounted on copper grids. Purified tobacco mosaic virus was used as an internal standard of measurement in this procedure.

Rotation Technique

The method of Markham et al. (1963) was used to determine icosahedral symmetry. Images of appropriate virus particles were enlarged from the negative with a Bessler 45 MCRX enlarger and projected onto photographic paper attached to a circular base. The center of rotation was fixed and the base was rotated five times at 72 degree increments for a five-step rotation or six times at 60 degree increments for a six-step rotation. Exposure time at each rotation was adjusted such that the final print was properly exposed.

Cell Culture

The monkey kidney cell line, TC7, a kind gift from Dr. J. C. Leong, Department of Microbiology, Oregon State University, was grown in 75cm^2 plastic flasks (Falcon) with Dulbecco's minimal essential media (DME) (Morton, 1970) supplemented with 10% (v/v) fetal bovine serum (FBS)(Grand Island Biologicals), 2mM glutamine, penicillin (50I.U./ml) and streptomycin (50µg/ml). After cells reached confluency, media with 1% FBS was used.

Chick embryo cell cultures were prepared as follows: 11 to 12-day old chicken embryos were removed aseptically from the shell, and the eyes, beak and shanks removed. The remaining tissues were

macerated with sterile scissors and washed with phosphate buffered saline (PBS) pH 7.1 until the supernatant was clear of cell debris and blood cells. The washed tissue was digested by stirring with 10 volumes of 0.15% trypsin for 10 minutes at 37 C. The supernatant from the first digestion was discarded. Digestion with trypsin was repeated until no tissue remained. One percent FBS was added to the cell suspension of each trypsinization to stop residual enzyme action. The pooled supernatants were filtered through a tissue sieve to remove large debris. The resulting cell suspension was centrifuged at 200 x g for 10 minutes and the packed cells resuspended in HLH with 0.06% $NaHCO_3$. The cells were washed twice and resuspended as described above. The viable cell count was determined and the cells were appropriately diluted in Eagle's Minimum Essential Median (MEM) with 5% FBS, 5% lactalbumin hydrolysate and 2% tryptose phosphate broth (Difco). Initial cell concentration was 5 x 10^5 cells/ml using 25 ml in each $75cm^2$ flask and 8 ml in a $25cm^2$ flask. Complete cell monolayers formed in 48 to 72 hours.

Cells were inoculated when 80% of the monolayer had formed. The inoculum consisted of one ml/$75cm^2$ flask or 0.3ml/$25cm^2$ flask of either stock virus or purified virus. The multiplicity of infection (MOI) was 0.1 or less. Cells were washed three times with HLH prior to inoculation. Virus was allowed to adsorb for 90 to 120 minutes at 27 C and the inoculum was replaced with MEM containing 0.5% FBS. After collection of culture fluids for titration, cultures were washed once with PBS prior to replacing the media.

Virus Growth in the Presence of BUDR

Cell cultures were treated with 20μg/ml of 5-bromodeoxyuridine (BUDR)(Sigma) one hour prior to inoculation with AEV. Similarily, cultures were treated with 20μg/ml BUDR plus 30μg/ml thymidine (Sigma). After virus adsorption, the BUDR and thymidine were again added to their respective cultures. Untreated cell cultures were inoculated for controls. At 2, 6, 8, 12, and 16 days post inoculation, the cell culture fluids were removed, stored at -70 C and fluids replaced with fresh media containing the appropriate compounds. Cell culture fluids were titrated for viral infectivity as above.

Radio-labelling of AEV with Nucleic Acid Precursor

Four to six days post inoculation, cell culture medium was replaced with two ml MEM supplemented with 0.25% FBS and containing 20 μCi/ml of either uridine [5-^{3}H] (ICN Pharamaceuticals Inc.) or thymidine (methyl-^{3}H) (ICN). Uninoculated cultures were similarily treated for controls. Medium containing the precursor was replaced every three to four days and the removed media pooled. The media were centrifuged at 3,000 x g for 30 minutes and the supernatant was concentrated by placing in dialysis tubing and covering with polyvinylpyrrolidone (Sigma). When the volume was reduced to three ml, the sample was centrifuged through the non-linear gradient. Fraction 2 from the nonlinear gradient was treated with nuclease and isopycnically centrifuged as above. Fractions of 0.3ml were collected and one ml of 0.01 M sodium pyrophosphate was added to each. After mixing, one

ml of 10% trichloroacetic acid (TCA) was added followed by 0.1ml of a 10mg/ml solution of bovine serum albumin (BSA) (Sigma). The TCA precipitable material was collected on GSB glass fiber filters (Whatman) under vacuum and washed with three volumes of 0.005M sodium pyrophosphate in 0.5N HCl. The filters were washed with one volume of 95% ethanol and allowed to dry at 37 C. The filters were counted for five minutes in Ready-Solv EP liquid scintillation cocktail (Beckman) with a Packard Tri-Carb liquid scintillation spectrometer.

The cell layers were examined in addition to the cell culture fluids. The monolayers were removed by trypsin digestion and were frozen at -20 C. After two cycles of freeze-thaw the suspension was sonicated for one minute at 60% capacity with a Biosonik IV (Bronwill Scientific). An equal volume of TTE was added to the sonicate which was shaken and centrifuged at 6,000 x g for 20 minutes. The aqueous layer was treated the same as the cell culture fluids above.

Nucleic Acid Extraction

All virus used for nucleic acid extraction was purified by the isopycnic gradient. Two methods were used to extract the nucleic acid from AEV. Detergent extraction was accomplished by heating AEV in TBS containing 1mM disodium (ethylenedinitrilo) tetraacetate (EDTA) (Baker) at 56 C in the presnece of 2% sodium dodecyl sulfate (SDS) (J. T. Baker). The nucleic acid was isolated by sucrose gradient centrifugation. Nucleic acid also was extracted by the phenol-SDS method of Bachrach (1960) modified as follows. An equal volume of buffer saturated phenol, made by dissolving approximately

8.8 grams crystallized phenol (Malinckrodt) in 1.2ml of TBS containing 1% SDS and 10mM EDTA, was added to the virus sample. The mixture was shaken for 10 minutes and the phases separated by centrifugation at 1,000 x g for 10 minutes. The aqueous phase was removed and extracted two more times. Residual phenol was removed from the aqueous phase by two extractions with peroxide free diethyl ether. Peroxides were removed from the ether by extraction with saturated ferrous sulfate solution (Scherrer, 1969). The ether was removed by bubbling nitrogen through the sample. Sodium acetate was added to 2% and the nucleic acid solution stored at -20 C.

Alternatively, after phenol extraction the nucleic acid was precipitated by adding two volumes of cold 95% ethanol and placing at -20 C overnight. One milligram of Type X transfer RNA (Sigma) was added as a carrier. The precipitate was collected by centrifugation at 30,000 g for 10 minutes. The nucleic acid pellet was resuspended in 200µl TBS containing 0.1% SDS and 1mM EDTA. The nucleic acid from Tobacco Mosaic Virus (TMV), obtained from the Department of Botany and Plant Pathology, O.S.U., was phenol extracted as above.

Chemical Analysis of AEV

Samples of AEV were analyzed for protein content by the method of Lowry *et al.* (1951) as follows: a solution of 1% $CuSO_4 \cdot 5H_2O$ was mixed with a 2% solution of $KNaC_4H_4O_6 \cdot 4H_2O$ just prior to use. The resulting solution was diluted 1 to 50 in a 2% Na_2CO_3 and 0.1M NaOH solution. Two and one half milliliters of this final solution was added to 25 to 50µl purified AEV. After 15 minutes at room temperature,

0.25ml of Folin and Ciocalteau phenol reagent (Steri-Kem Inc.) was added and the solution was mixed immediately. After 30 minutes at room temperature the optical density of the sample was read at 540nm in a Colman Model 55 spectrophotometer (Perkin-Elmer). Crystalline bovine serum albumin (BSA) (Sigma) was dissolved in distilled water, appropriately diluted and used to prepare a standard curve each time the assay was performed.

The orcinol colorimetric method as described by Shatkin (1969), was used to determine RNA concentration in AEV samples. Samples of AEV or phenol-extracted nucleic acid were hydrolyzed by heating in 0.5N perchloric acid at 70 C for 15 minutes. In the case of intact AEV, proteins precipitated by acid treatment were removed by centrifugation at 2,000 x g for 10 minutes. The orcinol reagent was prepared by dissolving 100mg of $FeCl_3 \cdot 6H_2O$ and 100mg orcinol monohydrate (Mallinckrodtt) in 100ml of concentrated HCl. To a one ml sample, one ml of reagent was added and the tube placed in a boiling water bath for 45 minutes (Albaum and Umbreit, 1947). The sample was cooled and the optical density read at 670nm against a water blank. A standard curve was prepared using yeast RNA (Worthington Biochemicals) hydrolyzed as above.

DNA concentration was determined by a modified diphenylamine procedure (Shatkin, 1969). The reagent was prepared by dissolving 0.75 grams of diphenylamine (Mallinckrodtt) in 50 ml of glacial acetic acid containing 0.75 ml of concentrated sulfuric acid. Just prior to use, 0.1ml of 1.6% aqueous acetaldehyde was added to each 20ml of reagent. Two ml of reagent was added to one ml of acid hydrolized

sample, incubated for 18 to 20 hours at 25 C and the optical density read at 60nm against an incubated perchlorate blank. Standard DNA solutions were prepared using calf thymus DNA (Worthington) dissolved in 5mM NaOH and hydrolyzed in an equal volume of 1N perchloric acid. A standard curve was prepared with the appropriate dilutions in 0.5N perchloric acid.

As a control for the chemical analyses, samples of purified tobacco mosaic virus were analyzed by the above tests.

Ultraviolet Absorbance of AEV

The ultraviolet (UV) absorbance profile of purified AEV was determined by scanning from 300nm to 215nm in TBS, pH 7.5 with a Cary 219 spectrophotometer. In addition, mixtures of BSA and yeast RNA containing 14%, 27% and 40% RNA were scanned and compared with the UV profile of AEV.

Sucrose Gradient Analysis of Nucleic Acid

Ribonuclease free sucrose (Grade I, Sigma) was dissolved in TBS containing 0.1mM EDTA and 0.1% SDS. In addition, 0.1% diethylpyrocarbonate (DEP) (Sigma) was added to inhibit nuclease activity (Solymosy *et al.*, 1968). Five to twenty percent gradients were used in all experiments. The gradient forming apparatus was constructed by connecting two plastic 10 x 75nm tubes at the base with plastic tubing. An outlet was made in one of the tubes and was connected to a peristalic pump with plastic tubing. This chamber held the higher

density sucrose. The gradient was formed as the two solutions were mixed while being withdrawn by the pump. Two hundred microliters of RNA solution was layered carefully on top of the gradient and centrifuged at 192,000 x g for 185 minutes in an SW 50.1 rotor. Tritium labelled 28 and 18S mammalian ribosomal RNAs, a generous gift of Dr. J. C. Leong, Department of Microbiology, O.S.U., were also layered on the gradient as sedimentation rate markers. Twenty drop fraction were collected from the bottom of the tube resulting in approximately 30 fractions. The percent sucrose of each fraction was determined with a Model 10406 refractometer (American Optical) calibrated for sucrose. The optical density of each fraction was determined at 254nm in an ISCO Model UA-5 absorbance monitor with a type-6 optical unit and microcuvett adaptor (Instrumentation Specialties Co.). Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer by counting a 100μl sample of each fraction for five minutes in Ready-Solv (Beckman). To determine relative composition, fractions included in a peak were combined and their absorbance was scanned from 300 to 215nm.

Gel Electrophoresis of AEV Nucleic Acid

Gel electrophoresis was used to determine the molecular weight of AEV nucleic acid. Ten centimeter agarose-acrylamide composite gels of 0.5% agarose (Seakem ME, Marine Colloids Inc.) and 2.4% acrylamide (Bio-Rad Laboratories) were prepared in 0.5mm I.D. glass tubes with buffer containing 40mM Tris, 20mM sodium acetate and 2mM EDTA, pH 7.5.

Polymerization was accomplished with 0.1% ammonium persulfate (Bio-Rad) and 0.01% N, N^1-Methylene-bis-acrylamide (Bio-Rad).

Two and one-half percent acrylamide gels containing 8M urea (Mallinckrodt), 20mM Tris-HCl and 2mM EDTA pH 7.5 were prepared according to Reijnders et al. (1973) and allowed to swell for 24 hours.

Electrophoresis was conducted at 5mA/tube for 30 minutes prior to sample application to remove polymerization catalyst and nuclease. The sample, in a 5% sucrose solution was applied with a micropipette in volumes varying 50µl/gel to 10µl/gel. Tritium labeled 28 and 18S ribosomal RNA and phenol-extracted TMS RNA were used as molecular weight markers. Electrophoresis was conducted at 5 C for acrylamide-agarose gels and at 40 C for urea gels by use of a constant current of 5mA/gel with a LKB Type 3290 B DC power supply. Samples were electrophoresed from 90 to 240 minutes and stopped when the dye band was 1cm from the bottom of the gel.

To visualize the bands, gels were removed from their tubes, fixed for 15 minutes in 5% acetic acid and stained for one to two hours in 0.2% methylene blue dissolved in buffer containing equal volumes of 0.4M sodium acetate and 0.4M acetic acid. Gels were also stained overnight in 0.05% toluidine blue solution in 5% acetic acid or stained for one hour with a 0.05% solution of 1-ethyl-2[2-(1-ethylnaphtho[1,2d]thiazolin-2-ylidene)-2-methylpropenyl] naphtho [1,2d] thiazolium Bromide ("Stains All") (Eastman Kodak) in a 50% formamide buffered to pH 7.3 with HCl. All gels were destained with distilled water.

The distance each band migrated from the top was measured and relative electrophoretic mobility determined by using the tracking dye front as a common reference in all tubes. Stained gels also were scanned at 550-570nm, depending on the stain used, with a scanning densitometer (Transidyne RFT General). Relative mobility again was determined using total gel scan and the peak location. Tritium labelled markers were located by slicing the gel into 2mm segments and counting in Ready-Solv (Beckman). Prior to counting, each gel slice was dissolved with 0.2ml of 60% perchloric acid and 0.4ml 30% hydrogen peroxide by heating to 30 C for three to four hours (Mahin and Lofberg, 1966).

RESULTS

Density of AEV

Virus purified by the non-linear gradient was centrifuged isopycnically and the gradient was fractionated and assayed for infectivity. Maximum infectivity corresponded to a density of 1.30-1.31g/ml (Figure 1). Minor infectivity peaks were evident at densities of 1.34 and 1.40.

Electron Microscopy

Micrographs of AEV stained with PTA and those fixed with OsO_4 followed by staining with UA showed that the virions had a hexagonal profile lacking an envelope (Figure 2). PTA staining failed to show any obvious surface detail while UVA staining gave better contrast and resolution of surface structure. The UA staining also showed a "core-like" positive staining of the center probably because of the affinity of UA for nucleic acids (Valentine, 1961). AEV purified by the non-linear gradient method apparently is free of major contaminants (Figure 2).

The replica technique was used to examine the structure of AEV. The electron micrographs reveal particles with a "raspberry-like" appearance (Figure 3). Three protrusions were discernable on the edges of several virus particles.

The size of AEV was determined by measuring virions prepared by the various staining methods. With PTA staining, the diameter was

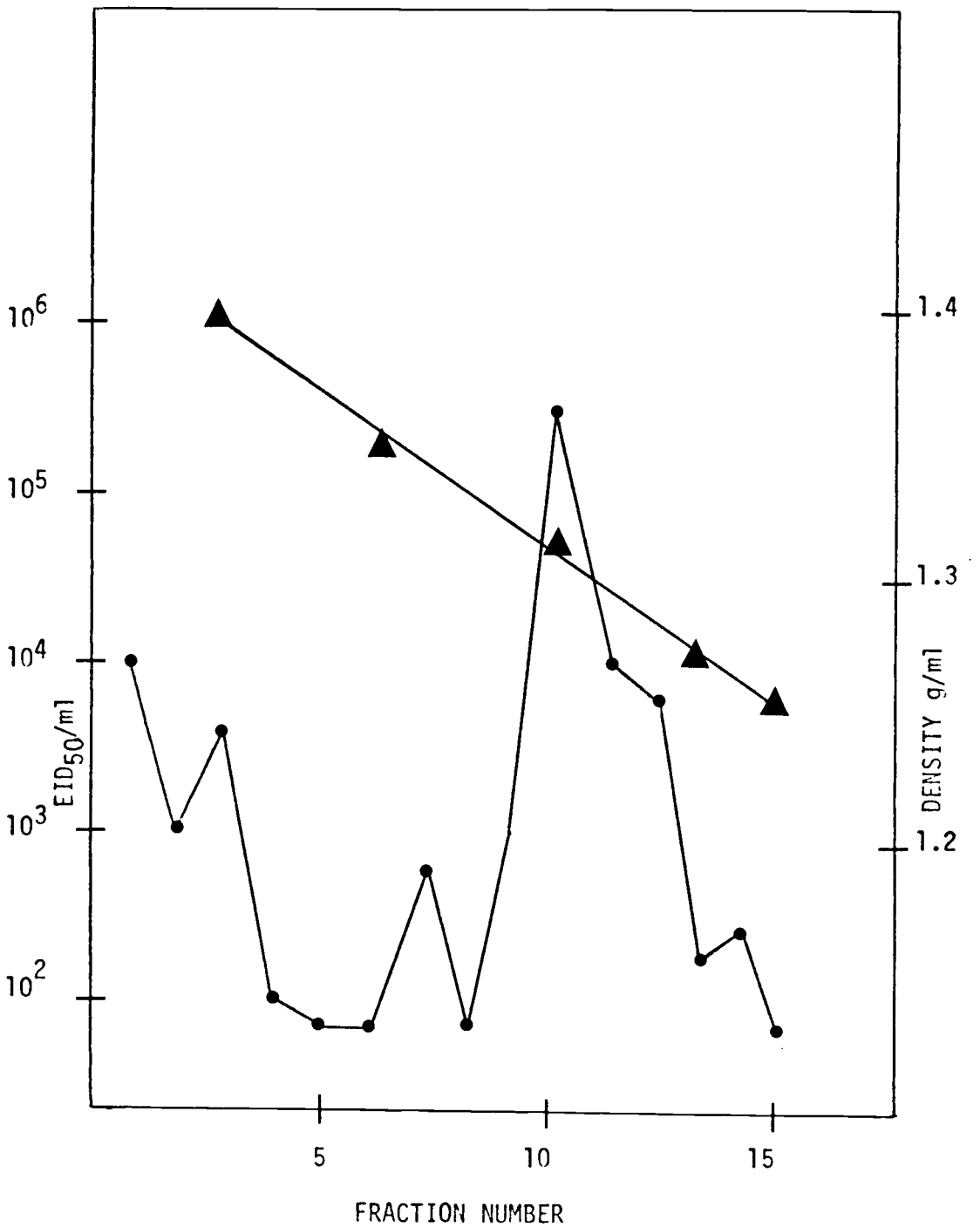

Figure 1. Infectivity (●——●) and density (▲——▲) of fractions after isopycnic gradient centrifugation of purified AEV in CsCl.

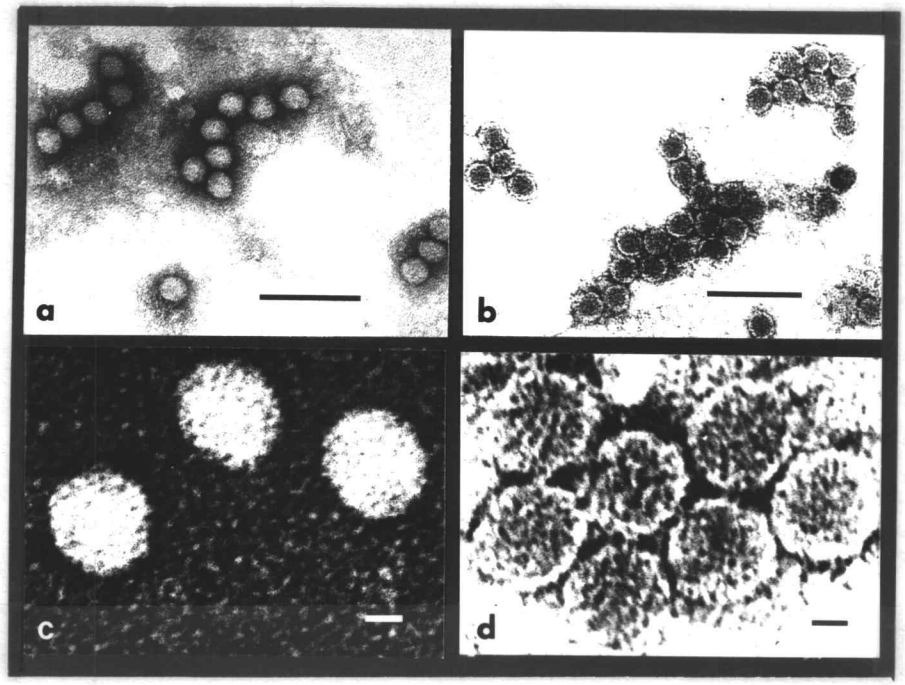

Figure 2. Electron micrographs of stained AEV: (a) and (c) negatively stained with PTA; (b) and (d) fixed with OsO_4 and positively stained with UA. Bar represents 100nm (a and b) or 10nm (c and d).

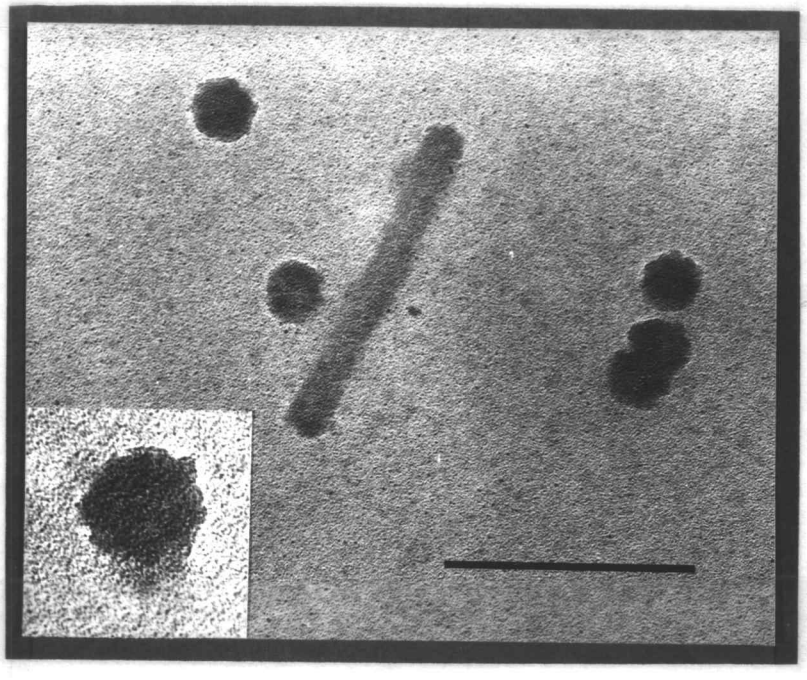

Figure 3. Electron micrographs of shadow-cast replica of purified AEV. For relative measurement purified TMV was included. The insert shows higher magnification. Bar equals 100nm.

27 ± 2nm (mean ± standard deviation) in comparison with the lattice-spacing of bovine catalase crystals. The virions which were fixed with OsO_4 and stained with UA had diameters of 30± 2nm, while the replica of AEV showed 26 ± 2nm after shadow-casting. The latter was based on a mean thickness (18nm) of TMV (Mattern, 1962a).

Krauss and Ueberschar (1966) examined AEV prepared by a chloroform extraction of infected chick brain. They proposed that AEV has icosahedral shape possessing 12 distinct capsomers. In Figures 2 and 3 none of the virus particles show the proposed structure. Accordingly, their method of preparing and staining the virus was followed conscientiously to reproduce their results. A typical example is shown in Figure 4. AEV prepared by their method failed to demonstrate particles having the proposed 12 capsomer structure. Virions had a similar surface structure to those shown in Figures 2a and c.

The purified AEV was treated with various agents, and their effects on viral morphology were examined. At pH 10.5 many AEV particles exhibited an increase in diameter (Figure 5a). Moreover, some had an empty capsid appearance while others showed varying types of partial degradation similar to those observed in poliovirus treated with cesium ions (Boublik and Drezeniek, 1977). On the other hand, the virions did not show any morphological alteration when kept at pH 4.5 (Figure 5b). Treatment with Triton X-100 resulted in complete degradation of the virus because no intact virions could be found after the treatment. Trypsin treatment produced no significant change in morphology. Mild urea treatment caused approximately a 30% increase in diameter (Figure 5c). Treatment of AEV with UA and Reynolds' lead

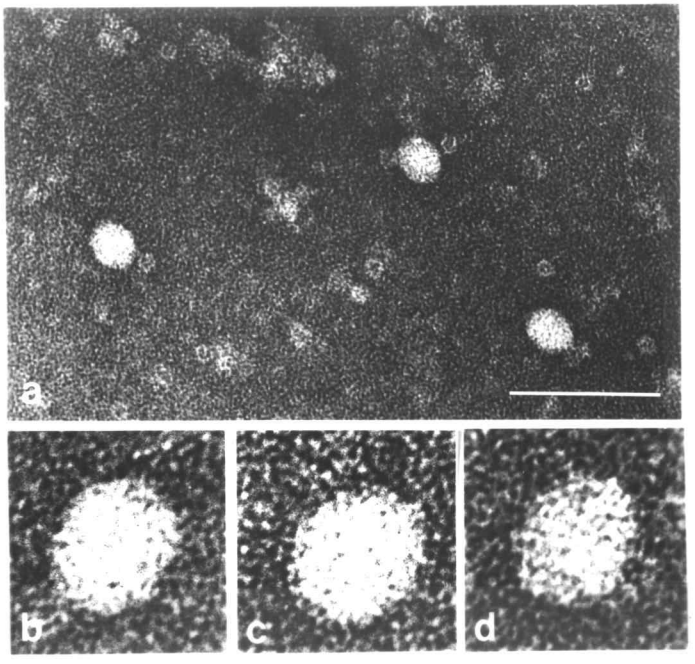

Figure 4. Electron micrographs of PTA-stained AEV particles found in sample prepared by chloroform-extraction of infected embryo brain; (b), (c), and (d) are enlargements. Bar equals 100nm.

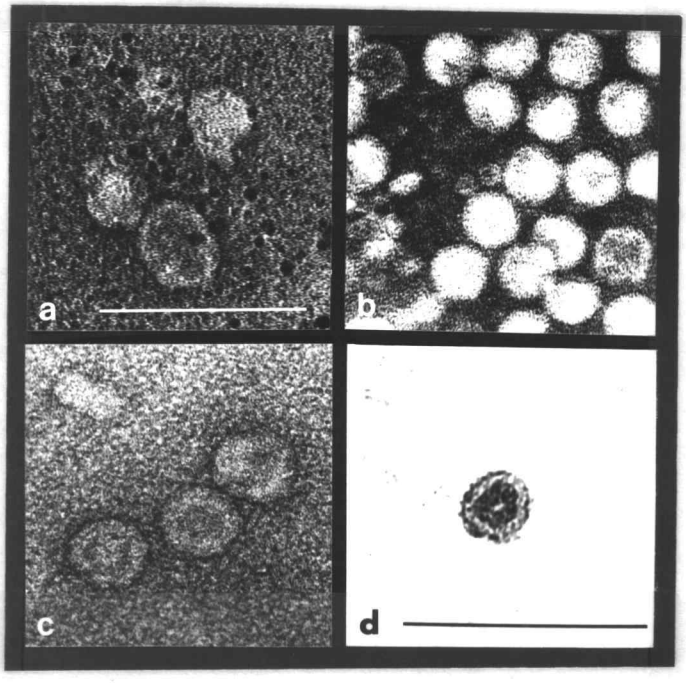

Figure 5. Electron micrographs of purified AEV treated with various agents; (a) at pH 10.5, PTA-stained; (b) at pH 4.5 PTA-stained; (c) treated with mild urea, PTA-stained; (d) treated with UA and Reynolds' lead cirtrate, successively. Bar equals 100nm.

citrate resulted in a typical dense positive staining of the nucleic acid core (Figure 5d). Wrigley (1969) suggested that "Afrin" may aid in identifying surface detail. Incubation of AEV with "Afrin" did not produce changes in morphology.

Rotation Technique

To determine icosahedral symmetry, rotations were performed on enlarged images of virions fixed with OsO_4 and stained with UA from the original photographic plate. In nearly all particles examined, centers reinforced by 5- and/or 6-step rotation were found. Figure 6 shows rotations of a representative particle which exhibits two centers of 5-fold symmetry. An original photograph is shown in duplicate (Figures 6a and e) with one of them indicating the centers of the rotations. When a 5-step rotation was performed around capsomer 1, it resulted in the clear reinforcement of a center of 5-fold symmetry (Figure 6b). Of interest is that capsomers immediately adjacent to the center and the next distant ones were clearly reinforced. The observation indicates that the capsomer 1 must be close to the center of the virion. When a 6-step rotation was performed around the same center, no reinforcement was observed (Figure 6f). Likewise, with respect to capsomer 2, 5-step or 6-step rotation (Figure 6c and g, respectively) indicated again a clear 5-fold symmetry.

Virion models consisting of 42 and 32 capsomers are displayed in Figure 6d and h, respectively, with corresponding centers of the rotations.

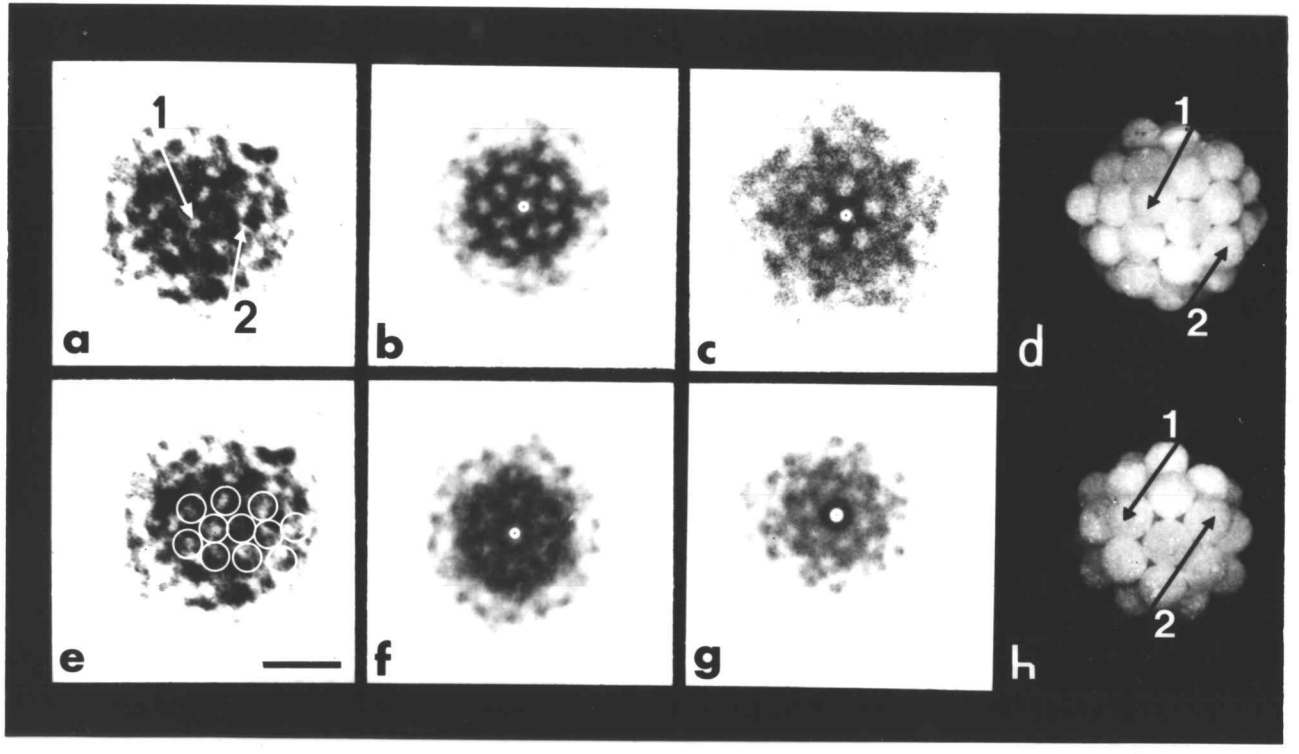

Figure 6. Photographs taken by rotating electron micrographs of UA-stained AEV by Markham's technique, demonstrating 5-fold symmetry. The particle was rotated with respect to centers 1 and 2 shown in (a); (b) and (f) are 5- and 6-step rotations, respectively, taking capsomer 1 as the center of rotation; similarily (c) and (g) are 5- and 6-step rotations with capsomer 2 as the center; (e) is a duplicate of (a) with suggested location of capsomers around the centers 1 and 2; (d) and (h) are models composed of 42 and 32 capsomers, respectively, with corresponding centers.

On another representative particle, 6-fold rotational symmetry was exhibited (Figure 7). Six-step and five-step rotations (Figure 7b and c, respectively) were performed on the center indicated (Figure 7a). Clearly, in addition to 5-coordinated capsomers, 6-coordinated capsomers also can be exhibited.

Growth of AEV in Cell Culture

Growth of AEV in a number of established cell lines was attempted (Abe, 1968) however, no growth was reported. Mancini and Yates (1968), on the other hand, showed increasing titers of AEV in primary chicken embryo cell fibroblasts. In this study, whole embryo cell cultures were inoculated with one ml of virus at 10^3 EID_{50}/ml, an MOI of approximately 0.001. Cell culture fluids from four flasks were collected at 1, 4, 8 and 12 days post inoculation, pooled and assayed for infectivity. All cultures were washed with PBS between fluid collections. As shown in Figure 8, the titer increased from $10^{0.7}$ to $10^{2.4}$ $EID/_{50}/$ ml by day nine, and dropped to $10^{1.7}$ EID_{50}/ml by day 12.

Halpin (1966) has reported substantial growth of AEV in monkey kidney cell culture, however, these preliminary results were not confirmed. In an attempt to examine growth in monkey kidney cells, TC7 cultures were inoculated with AEV. Cell cultures inoculated at an initial titer of 10^3 EID_{50}/ml showed no cytopathic effect or inclusions during a 16 day incubation period. Cell culture media was assayed every fourth day after which the cells were washed and fresh medium was added. By the 16th day post inoculation, no viral infectivity was detectable.

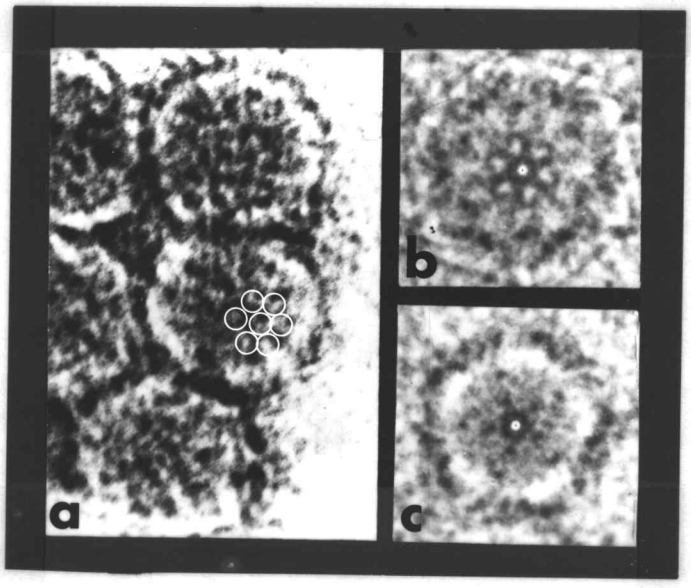

Figure 7. Photographs of rotated electron micrographs of AEV similar to Figure 6 but showing 6-fold symmetry; (b) and (c) are 6- and 5-step rotations, respectively, of the center indicated in (a).

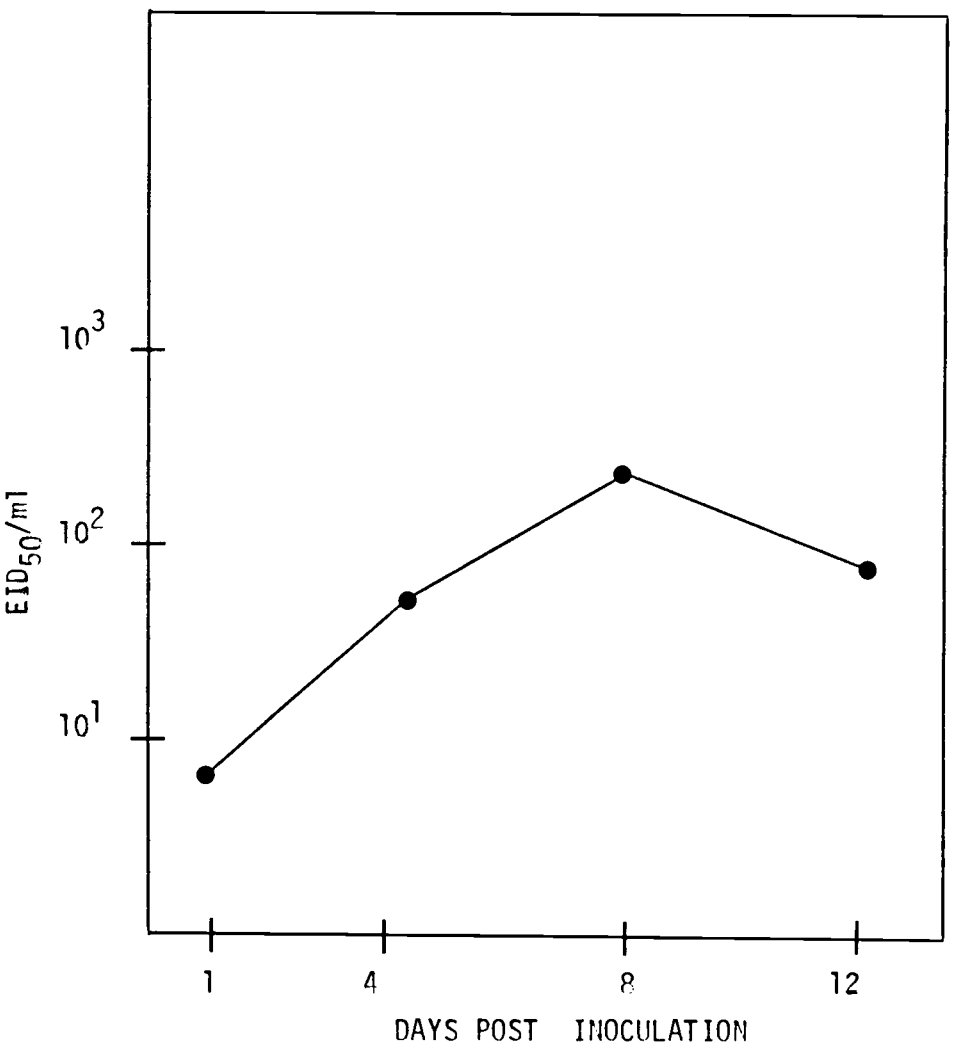

Figure 8. Growth of AEV in chick embryo cell culture indicated by the median embryo infective dose (EID_{50}) per milliliter of culture fluid at various times post inoculation.

In another experiment, the growth of AEV in cell culture was examined in the presence of an inhibitor of DNA replication, BUDR. The results of growth in the presence of BUDR and BUDR plus thymidine are shown in Figure 9. These results are the averaged values from duplicate cultures. No significant effect on virus growth was evident over the controls in the BUDR treated cultures. The addition of thymidine, however, resulted in a slight increase in virus production by 16 days.

Viral Nucleic Acid

Several methods were used to determine whether AEV has an RNA or DNA genome. Attempts were made to label the viral nucleic acid in cell culture with radioactive pyrimidine precursors. AEV was grown in the presence of ^{3}H-uridine and ^{3}H-thymidine and the culture fluids were concentrated and centrifuged as previously described. In the uridine labelled cultures, a peak of TCA precipitable radioactivity was detected that corresponded to the determined location of AEV in the isopycnic gradient (Figure 10). In repeated experiments, the peak was consistently at a density of approximately 1.31. There was no similar peak of radioactivity from those cultures grown in the presence of tritiated thymidine. As shown in Figure 10, uninoculated cultures incubated in the presence of ^{3}H-uridine showed no peak of activity.

To insure that the radioactivity present in the fractions containing the peak was not due to cellular RNA adsorbed onto the virus particles, uninoculated cultures were incubated with ^{3}H-uridine

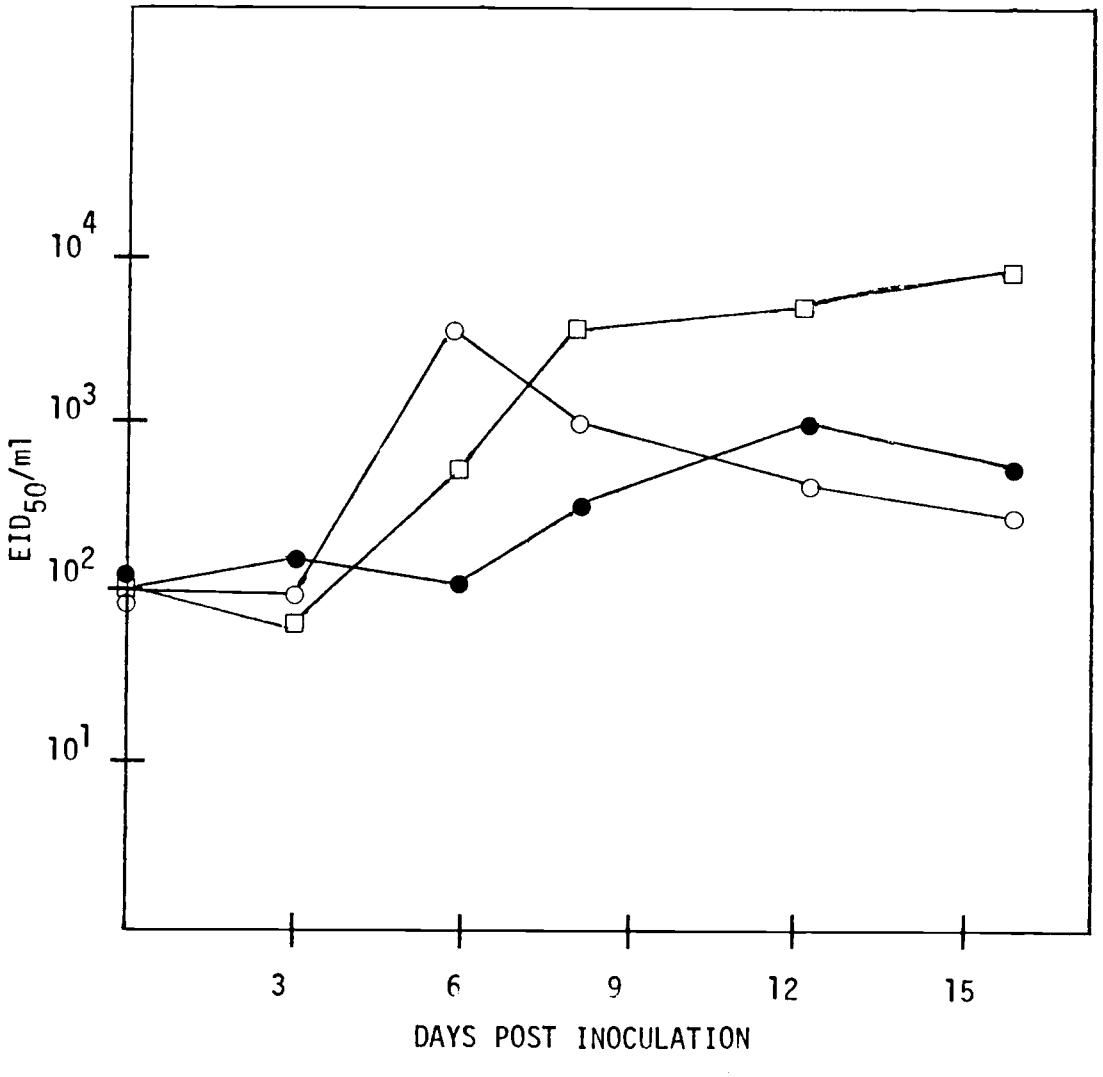

Figure 9. Effect of BUDR on the replication of AEV in cell culture. Untreated control culture (●——●); BUDR (○——○); BUDR and thymidine (□——□). The extent of replication was determined by the median embryo infective dose (EID_{50}) per milliliter of culture medium.

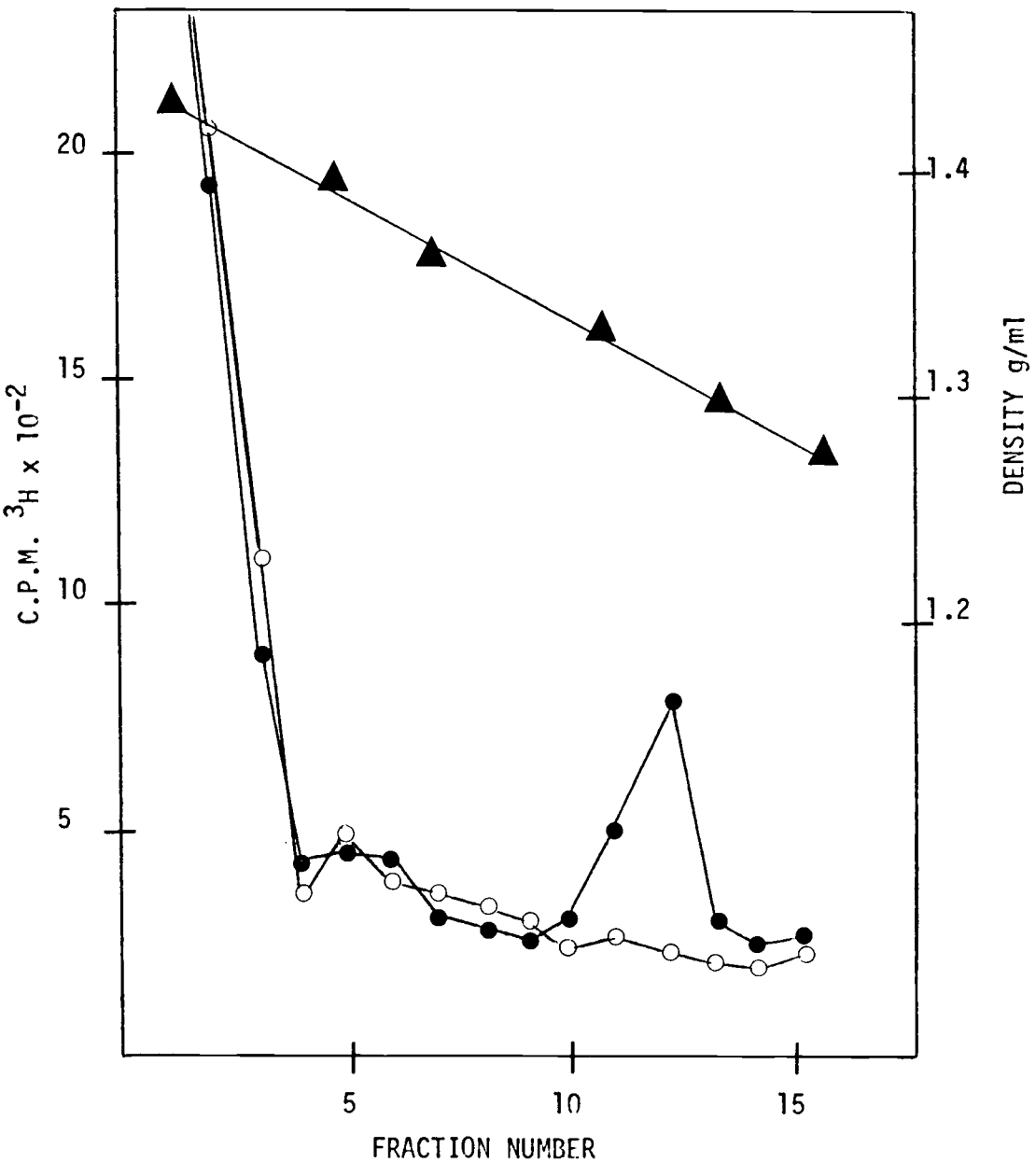

Figure 10. Acid precipitable radioactivity from an isopycnic gradient of cell culture fluids from infected cells grown in the presence of ^{3}H uridine (●——●) and fluids from uninfected cells grown in the presence of ^{3}H uridine (○——○). Density (▲——▲) was determined by weight.

exactly as the inoculated cultures. At the end of the incubation period, 100µl of purified virus (10^7 to 10^8 $EID/_{50}$/ml) was added to the cell culture fluids which were then concentrated and centrifuged as before. A visible virus band was evident in the isopycnic gradient. However, there was no TCA precipitable radioactivity corresponding to the location of this band.

To determine the type of viral nucleic acid by direct chemical means, purified samples of AEV were assayed for protein, RNA and DNA content by Lowry's method of protein determination, the orcinol and the diphenylamine colorimentric methods, respectively. The exact concentration of BSA, which served as a primary standard for Lowry's method, was determined by nitrogen analysis (Campbell et al., 1974). The results, shown in Table I, indicated that the virus contained an RNA genome. Virus purified by the nonlinear gradient was not of sufficient purity for accurate chemical analysis because both RNA and DNA were present in the fraction. Isopycnically purified virus samples, on the other hand, repeatedly contained only RNA and proteins.

In separate experiments, nucleic acid was extracted with phenol-SDS from isopycnically purified AEV and assayed for the type of nucleic acid by the above methods. By comparison with RNA determinations on the perchloric acid extracts of AEV, 51% of the nucleic acid was extracted by the phenol method. In two separate experiments, chemical analysis yielded the following values: 18µg/ml RNA and 121µg/ml protein and 14µg/ml RNA and 81µg/ml protein. These gave RNA percentage values of 12.9 and 14.1, respectively.

TABLE I. Chemical Analysis of AEV. Analysis was performed on acid hydrolyzed samples of purified virus. Protein content was determined by Lowry's method. The orcinol and diphenylamine methods were used to assay RNA and DNA content, respectively.

Sample	Protein (μg/ml)	RNA (μg/ml)	DNA (μg/ml)	Percent RNA	Percent DNA
		NONLINEAR GRADIENT PURIFIED			
1	1050	146	352	9.6	22.7
2	251	73	159	15.0	32.9
		ISOPYCNINC PURIFICATION			
3	165	62.0	0	27.3	0
4	113	47.1	0	29.4	0
5	88.9	27.5	0	23.6	0
6	106	43.2	0	29.0	0
7	82.1	28.8	0	26.0	0

In another attempt to confirm the nucleic acid type, 100μl nucleic acid, extracted by the thermal-SDS method, was treated with either 100 units of RNAse or 50 units of DNAse. After centrifugation through a 5 to 20% sucrose gradient, the location of the nucleic acid was determined by the optical density of gradient fractions. Ribonuclease treatment resulted in the loss of the major nucleic acid peak present in both the untreated control and the DNAse treated sample (Figure 11).

Using the method of McEwen (1967) the sedimentation coefficient of the major peak was calculated to be 32S. It should be noted that minor peaks are evident in the control but are absent or decreased in the nuclease treated samples. These peaks had sedimentation coefficients, from higher to lower sucrose percentages, of 20, 12, and 5S.

Figure 12 depicts the results of a similar experiment in which phenol-extracted nucleic acid was treated with the nucleases. Again the nucleic acid peak present in the DNAse treated sample was absent after RNAse treatment.

To further identify the minor peaks observed in the sucrose gradient with the thermal-SDS extracted AEV (Figure 11), preparations again were centrifuged but with the sedimentation rate markers. Figure 13 shows two major peaks, one at approximately 32S and another at 20S. To determine the relative composition of each peak, the 260/280 ultraviolet absorbance ratio of fractions from each peak was examined. Yeast RNA has a ratio of 2.04 while the protein enolase has a ratio of 0.57 (Segal, 1976). These values were used subjectively as indicators of peak composition. The 32S peak was identified

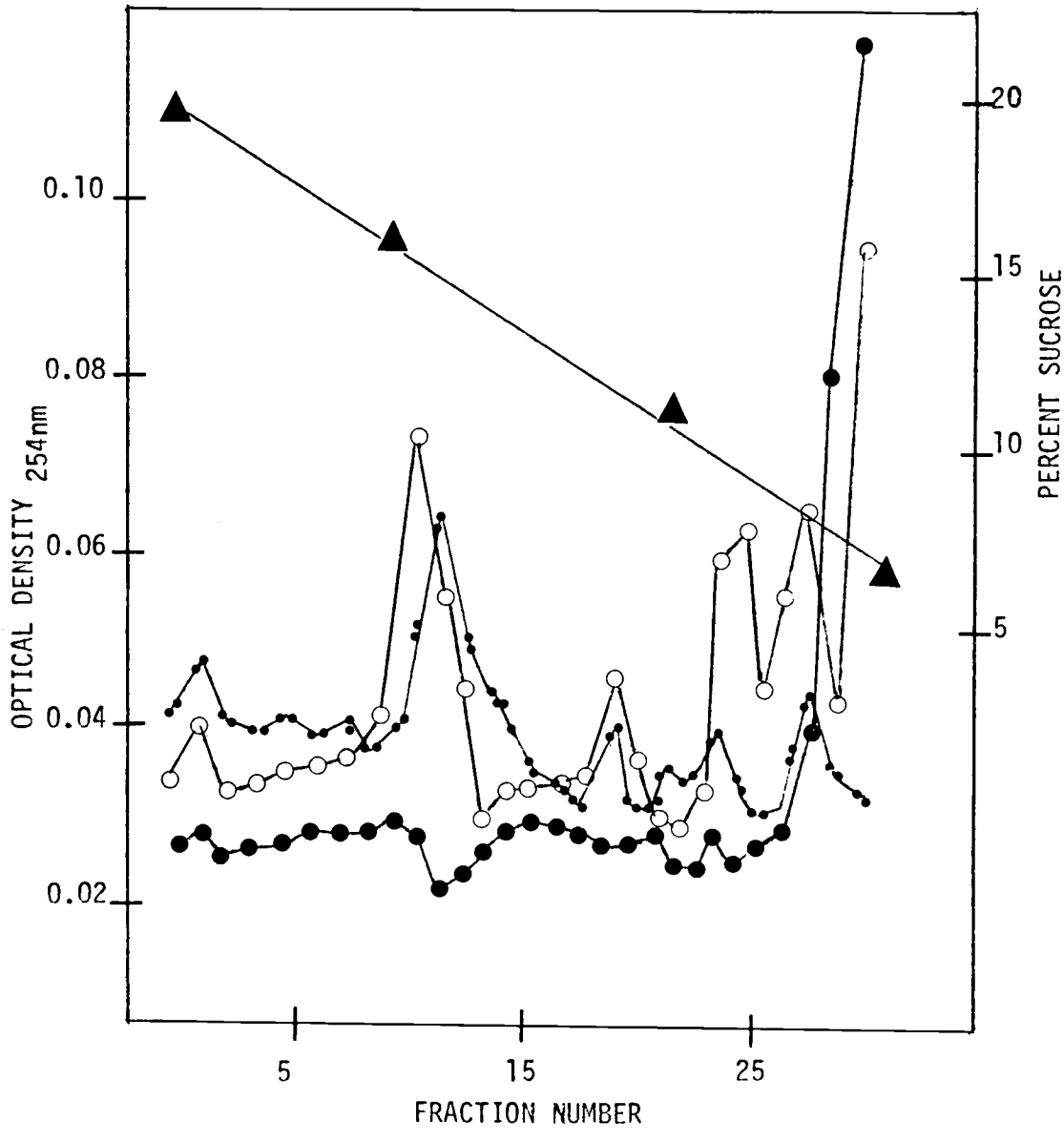

Figure 11. Sucrose gradient centrifugation of nuclease treated AEV nucleic acid extracted by the thermal-SDS method. Untreated control nucleic acid (○——○); DNAse treated (••——••); RNAse treated (●——●). Peak location was determined by the optical density of gradient fractions. Percent sucrose (▲——▲) was determined by refractive index.

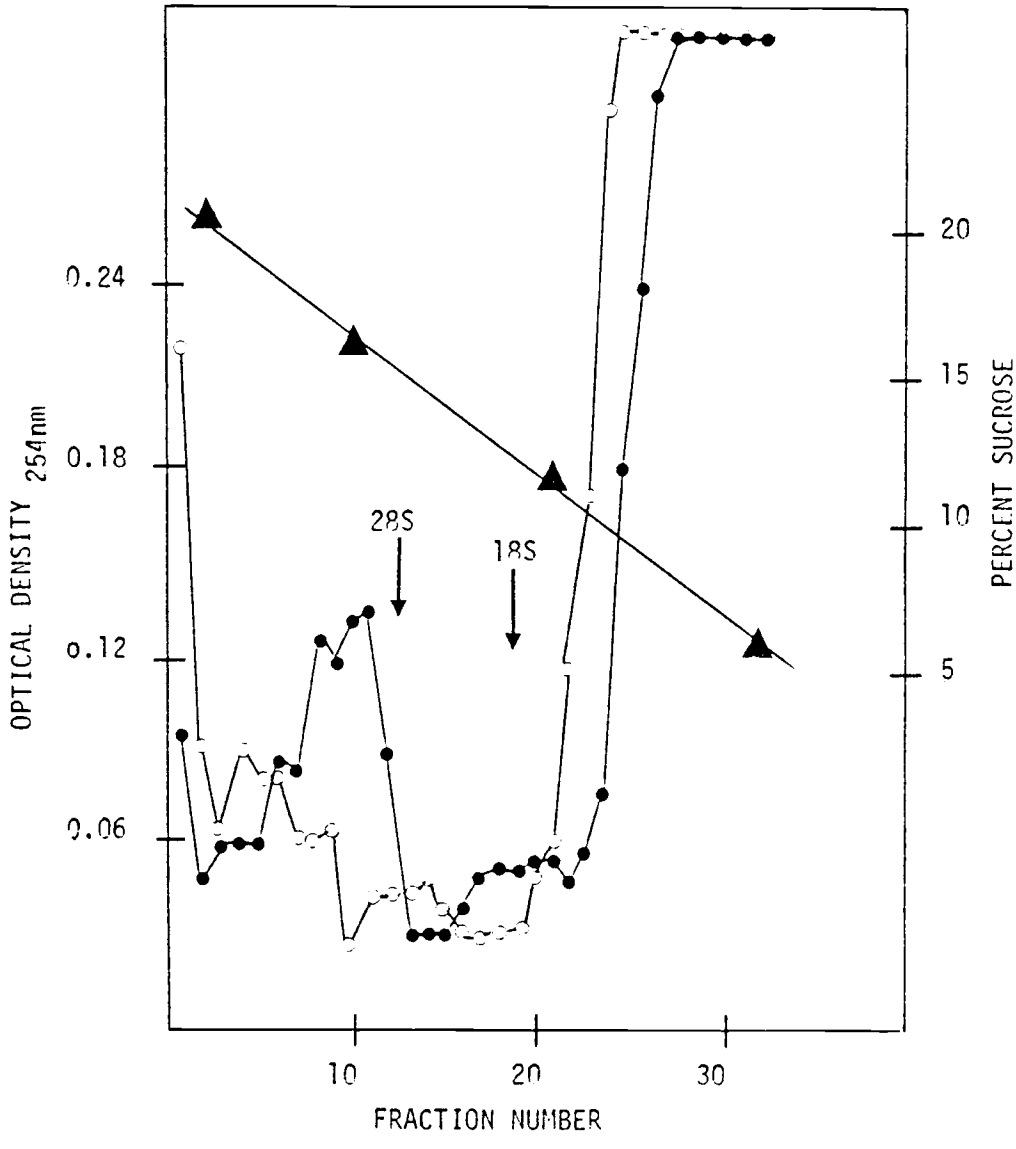

Figure 12. Sucrose gradient centrifugation of nuclease treated viral nucleic acid extracted by the phenol-SDS method. DNAse treated (●——●); RNAse treated (○——○). Location of tritium labelled 28 and 18S ribosomal RNAs used as markers is indicated. Peak location was determined by optical density. Sucrose percent (▲——▲) was determined by refractive index.

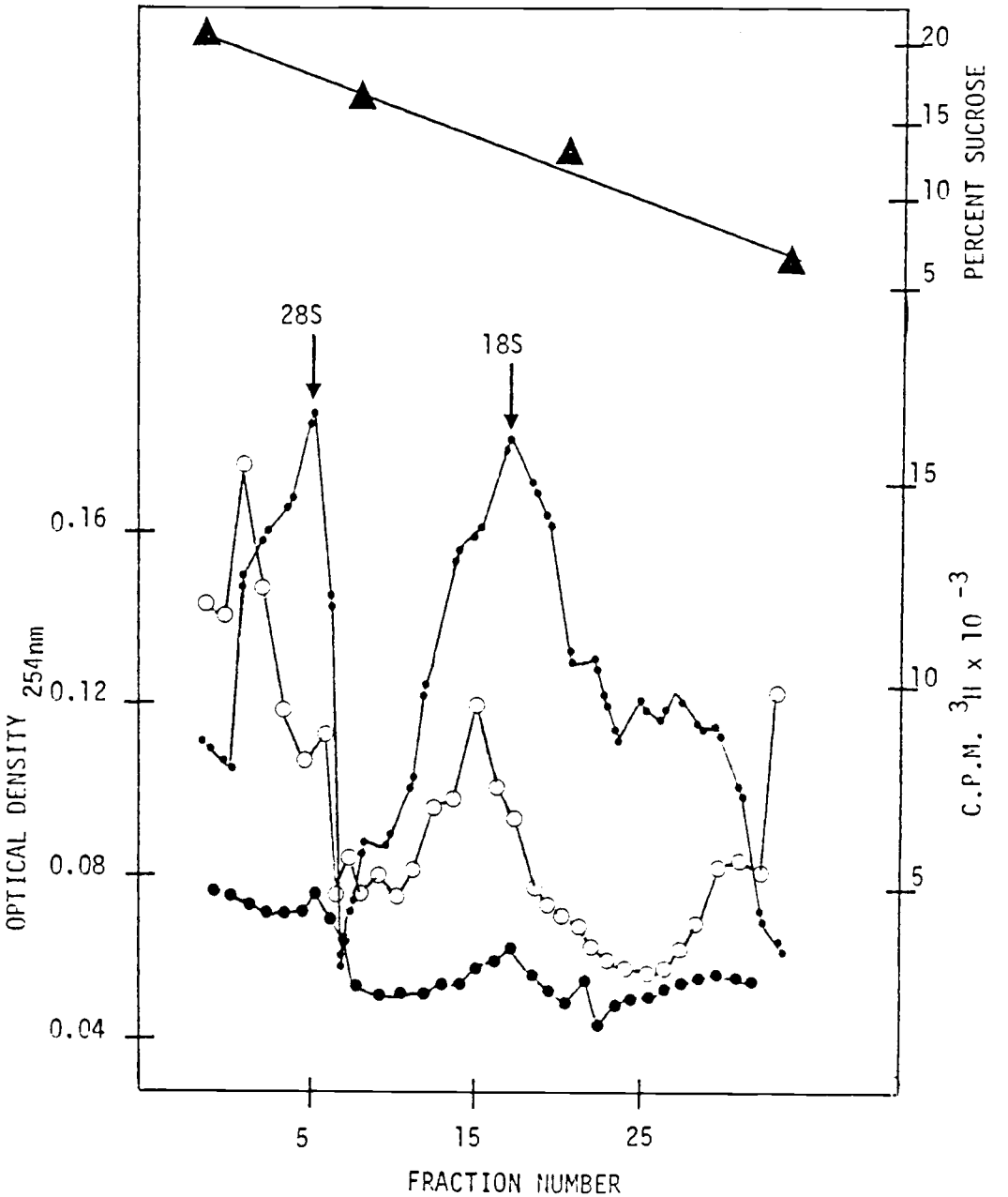

Figure 13. Sucrose gradient centrifugation of thermal-SDS extracted viral nucleic acid with tritium labelled ribosomal RNA markers. Viral nucleic acid, determined by optical density (○——○); 28 and 18S ribosomal RNAs, determined by radioactivity (••——••); 28 and 18S ribosomal RNAs, determined by optical density (●——●). Sucrose percentage (▲——▲) was determined by refractive index.

as nucleic acid while the 20S peak was of protein. Optical density measurements of fractions from a gradient containing only the tritium labelled markers indicates that the markers are not contributing to the observed optical density in gradients containing both sample and marker (Figure 13).

Physical Characteristics of the Virion and Viral Nucleic Acid

From the chemical data (Table I), the mean percentage of nucleic acid in the virion was calculated to be 27.1 for the isopycnically purified virus. TMV RNA was subjected to the chemical analysis. The virion contained 5.1% RNA which compares well with the reported value of 5.0% (Gibbs and Harrison, 1976) indicating the relative accuracy of the method for determining percentage values.

To further confirm the percentage value for AEV, ultraviolet scans of various percent combinations of yeast RNA and BSA were compared with the scan of AEV. As indicated in Figure 14, of the percents examined, the absorbance profile of AEV and the 27% RNA solution are the most similar both with a minimum at 238nm and similar optical density at 215. All samples had a maximum at 259nm.

From the UV scans of purified virus along with the protein concentration values determined by the Lowry's method, the extinction coefficient of AEV ($E^{1\%}$) at 260nm ranged from 70 to 81 with a mean value of 74. Values obtained from ultraviolet absorption spectra were corrected for light scattering as described by Burness (1970).

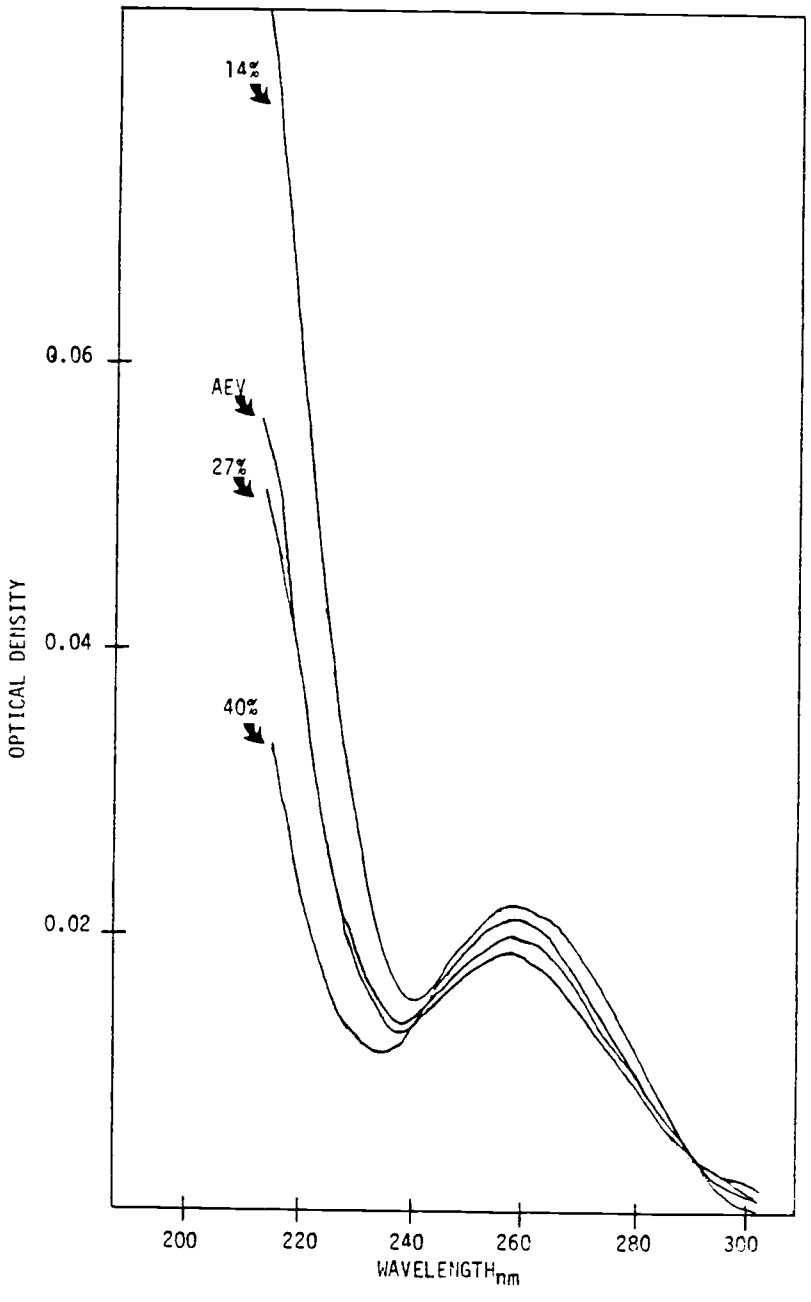

Figure 14. Ultraviolet scans of purified AEV and several combinations of yeast RNA and BSA. Values indicated are RNA percentages.

The sedimentation coefficient of the nucleic acid was determined in neutral sucrose, 0.15M NaCl, by comparison to 28 and 18S ribisomal RNAs and TMV RNA which has reported sedimentation coefficients of 26S, 27.9S (Boedtker, 1968) and 27S (Granboulan and Girard, 1969). The nucleic acid of AEV and TMV RNA were layered onto separate 5 to 20% gradients along with the 28 and 18S tritium labelled markers. The location of AEV and TMV nucleic acids were determined by the optical density of gradient fractions. To insure that the tritium labelled markers did not contribute to the observed optical density, these markers were centrifuged in a separate tube and the optical density of the gradient fractions determined. The results showed that AEV sedimented about 1.2 times faster than TMV RNA (Figure 15). The sedimentation coefficient of the sodium nucleic acid, estimated by the method of Martin and Ames (1961), ranged from 32.3 to 35.4 with a mean value of 33.6. The tritium labelled markers did not contribute to the optical density as indicated in Figure 15.

Attempts were made to determine the molecular weight of the viral nucleic acid with polyacrylamide gels containing urea and with agarose-acrylamide composite gels. Reijnder et al. (1973) indicated that urea gels were more accurate for determining molecular weights of RNA because under these denaturing conditions, conformation does not influence the migration. However, nucleic acid electrophoresed on urea gels resulted in diffuse bands which made it difficult to determine accurate electrophoretic mobility. Therefore, these gels were not used for the determination of molecular weight.

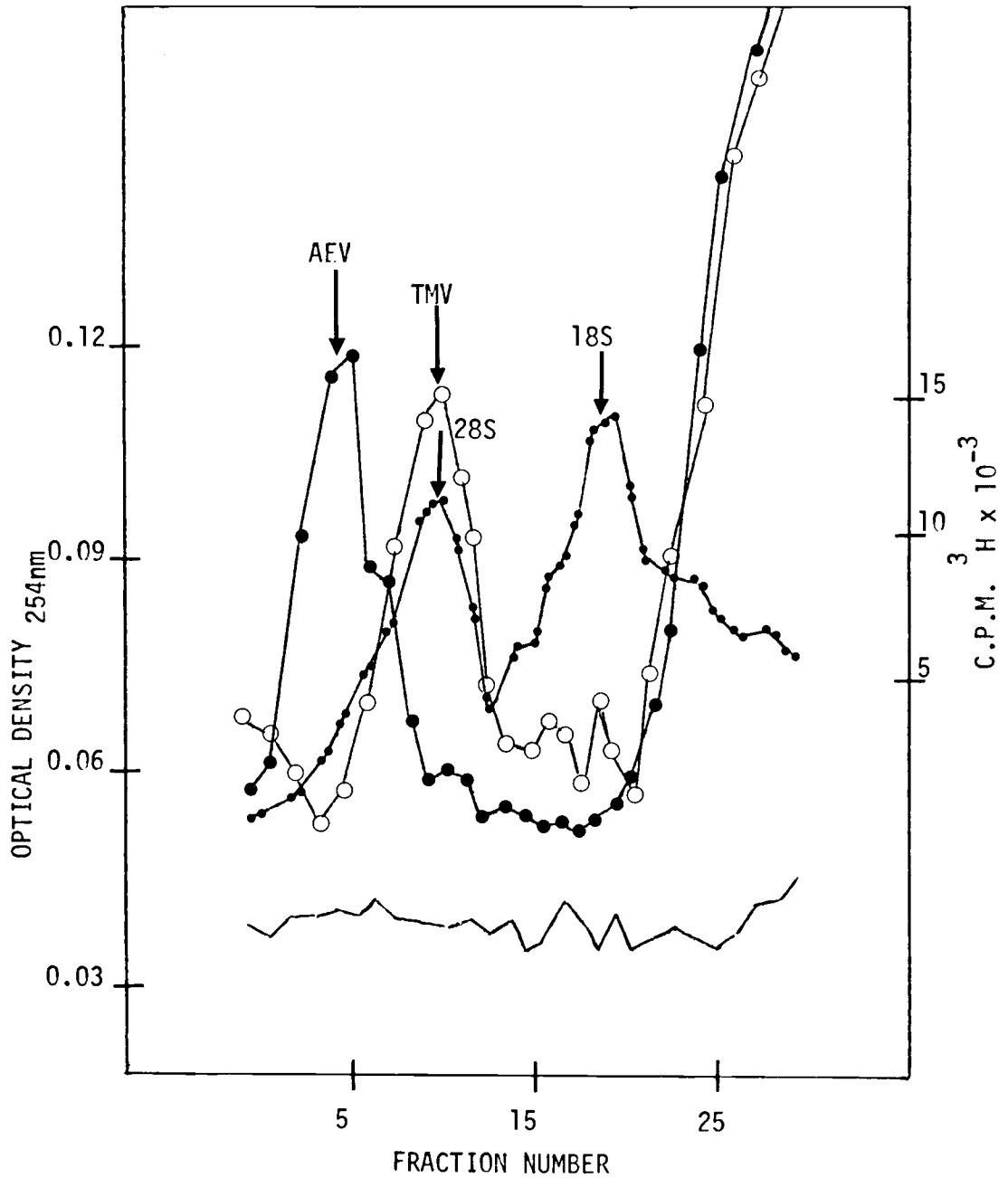

Figure 15. Sedimentation behavior of AEV nucleic acid in comparison to TMV RNA and 28 and 18S ribosomal RNAs. Samples were centrifuged through a linear 5 to 20% sucrose gradient. AEV nucleic acid (●——●); TMV RNA (○——○); 28 and 18S tritium labelled ribosomal RNAs (••——••); Optical density of ribosomal markers (———).

Viral nucleic acid, subjected to electrophoresis on agarose-acrylamide composite gels resulted in narrow bands and were more suitable for molecular weight determination. Following electrophoresis, the gels were stained and scanned with a densitometer. In relation to the tritiated markers, AEV exhibited a slower electrophoretic mobility than TMV (Figure 16). There was considerable degradation of AEV nucleic acid as indicated by the absorbance increase after the main peak. This was likely because of degradation during the extraction procedure.

The location of the AEV and TMV peaks in relation to each other was determined by aligning the 28 and 18S markers in each gel. To minimize error in determining the relative location of the TMV and AEV nucleic acid bands by this method, these two nucleic acids were electrophoresed in the same gel. Photometric scanning detected two separate peaks.

A plot of the relative mobility of the markers versus the log of their molecular weights (Figure 17) gave a linear plot as expected (Loening, 1969). The molecular weight values used for the marker RNAs were 2.0×10^6 daltons for TMV and 1.68 and 0.67×10^6 daltons for the 28 and 18S ribosomal RNAs, respectively. The results of three determinations of the molecular weight of AEV nucleic acid gave values of 2.26, 2.30, and 2.38×10^6 daltons. The mean value was 2.31×10^6 daltons.

The molecular weight of the viral nucleic acid also was estimated from its sedimentation coefficient. An equation:

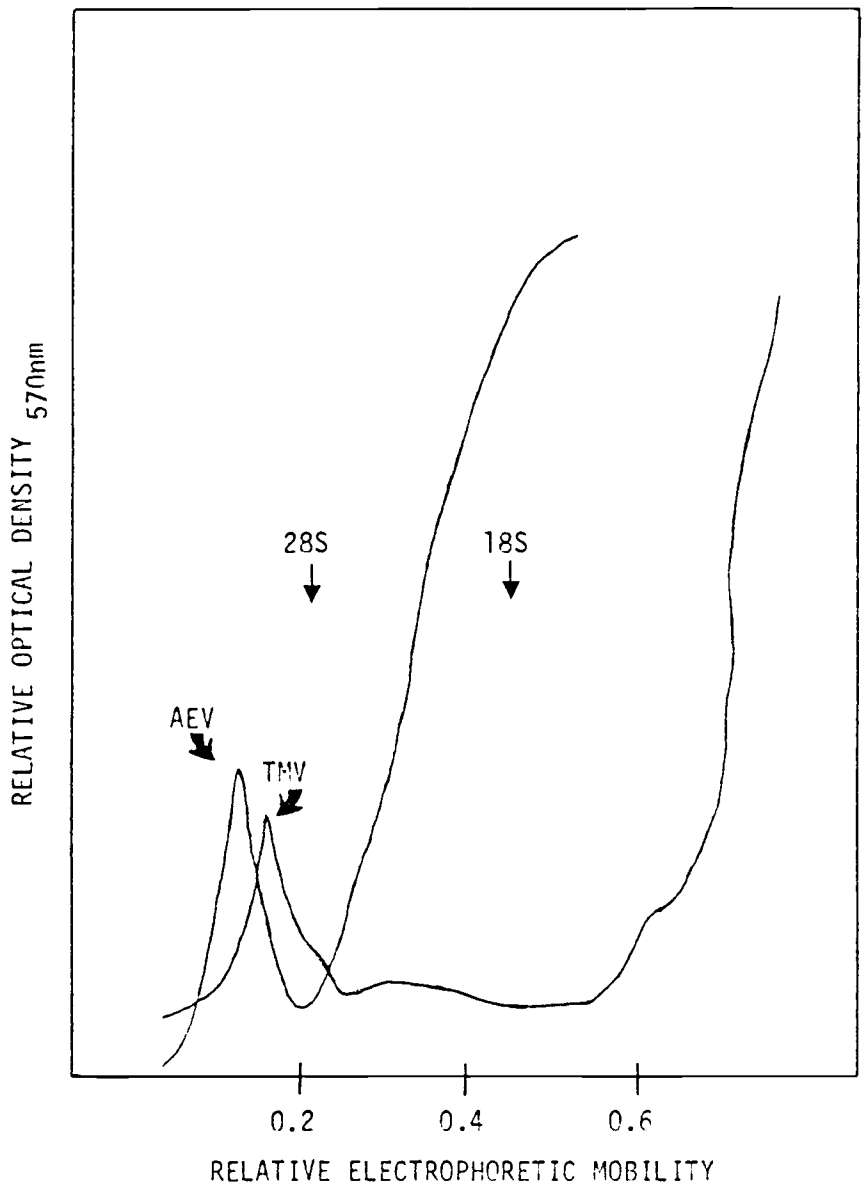

Figure 16. Electrophoretic mobility of AEV nucleic acid on toluidine stained agarose-acrylamide gels relative to TMV RNA and 28 and 18S ribosomal RNAs. Peak location was determined by photometric scanning. Ribosomal RNA location was determined by radioactivity of gel slices.

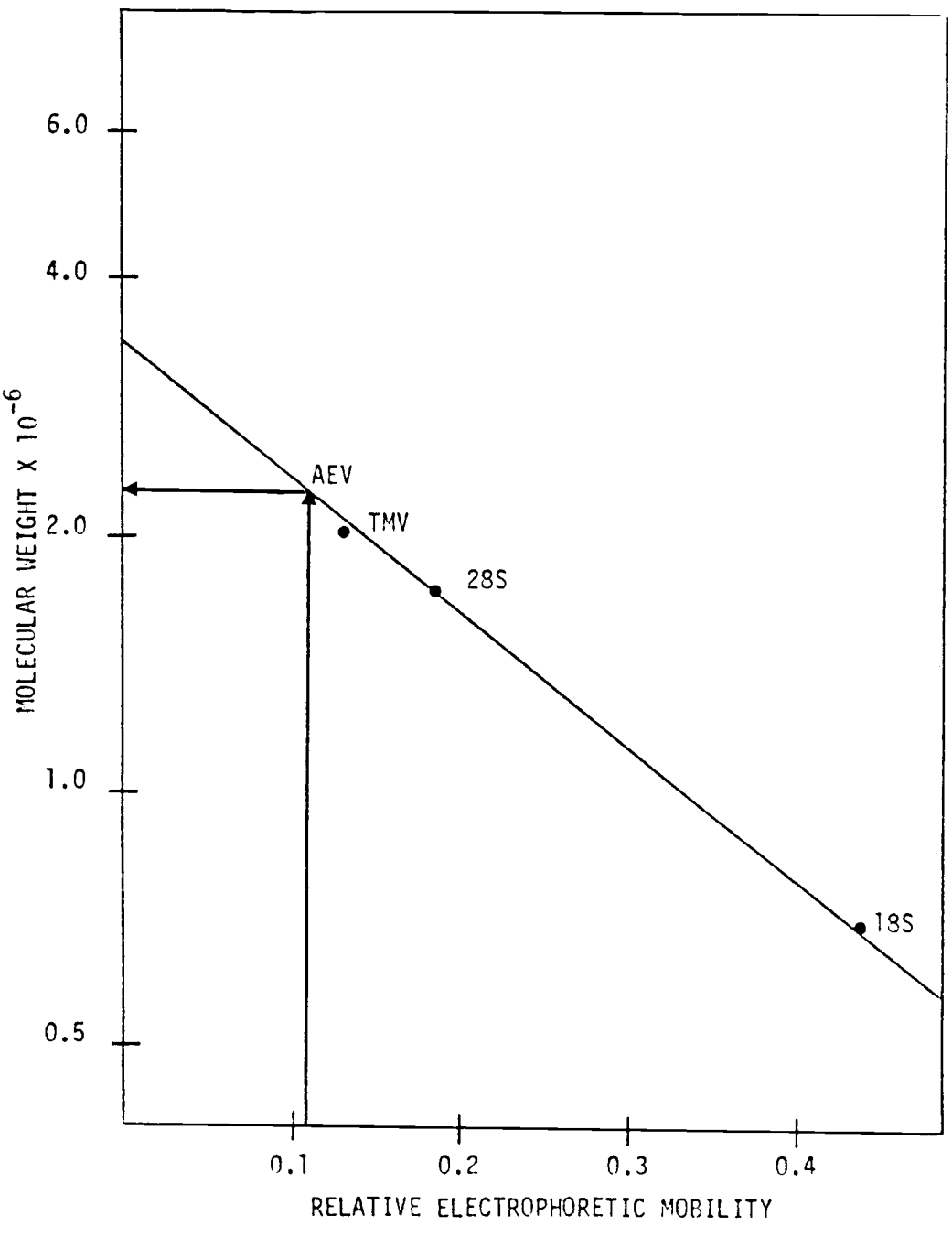

Figure 17. Plot of relative electrophoretic mobility vs molecular weight (log scale) for TMV RNA and 28 and 18S ribosomal RNAs. The linear plot was used to estimate the molecular weight of AEV nucleic acid from its relative electrophoretic mobility.

$$M = 14{,}300[s_{20,W}(\eta)^{0.33}]^{1.5}$$

where $s_{20,W}$ is the sedimentation coefficient in Svedberg units (10^{-13} seconds) and η is the viscosity of the nucleic acid, was derived empirically for determining the molecular weight of RNA (Boedtker, 1968). A value of 2.4×10^6 daltons was calculated using a value of 33.6S and the viscosity of TMV RNA (Boedtker, 1959), assuming the viscosity of AEV nucleic acid and TMV RNA are similar.

With the results of the size determination, virion sedimentation coefficient of 148S (Gosting, 1977), and the percent nucleic acid, the molecular weight of the virion was established using the equation (Svedberg and Pedersen, 1940):

$$M = RTs/D(1 - \bar{\nu}\rho)$$

where M = molecular weight, R the gas constant of 8.314×10^7 erg/deg/mole, s the sedimentation coefficient, T the absolute temperature of 293.2K, ρ the solution density of 0.998 g/ml at 20 C, $\bar{\nu}$ the partial specific volume of AEV and D the diffusion coefficient. A value of 7.6×10^6 dalton was obtained. The partial specific volume $\bar{\nu}$ was established knowing that the virion contained 27.1% RNA and 72.9% protein. These are assumed to have individual partial specific volumes of 0.55 and 0.74ml/g, respectively (Markham, 1967), giving a partial specific volume for the virus = (.271 x .55) + (.729 x .74) = .6885ml/g. The diffusion coefficient was estimated using the Einstein equation (Ackers and Steere, 1967):

$$D_{20W} = \frac{2.13 \times 10^{-5} \text{ cm}^2/\text{sec}}{a}$$

where (a) is the molecular radius. Using a mean diameter of 280 Å for AEV the diffusion coefficient was calculated to be 1.52×10^{-7} cm^2/sec.

Using a different method, the molecular weight of the virion was calculated to be 8.5×10^6 daltons. This value was calculated by dividing the nucleic acid molecular weight by its percentage in the virion.

DISCUSSION

One of the purposes of the present study was to determine the viral classification of AEV. The results of previous studies indicated that AEV belonged in either the picornavirus family, likely in the Enterovirus genus, or in the parvovirus family (Gosting, 1977; Butterfield et al., 1969; Krauss and Ueberschar, 1966; Buelow, 1964). Butterfield et al. (1969) claimed that AEV belonged in the Enterovirus genus on the basis of resistance to DNAse and a slight sensitivity to RNAse but, this classification was not widely accepted because of crude methodology. Certain other characteristics also suggested an enterovirus classification. The sedimentation coefficient of 148S (Gosting, 1977) compared well with reported values for picornaviruses of 140 to 160S (Brown and Hull, 1973; Bachrach et al., 1964), when compared with atuonomous parvovirus sedimentation coefficients of 105 to 122 (Salzman, 1977). The density of AEV, 1.30 to 1.32, when compared with values of 1.33 to 1.34 for enteroviruses (Newman et al., 1972) and 1.38 to 1.42 for parvoviruses (Salzman, 1977) was lower than both groups but comparable to the enteroviruses. Even though these characteristics suggested of an enterovirus classification, definitive proof rested on the determination of nucleic acid type. Results of the present study indicated an RNA genome which suggested that the virus was a member of the family Picornaviridae.

The buoyant density of AEV was reported previously to be 1.31 to 1.32g/ml (Gosting, 1977) and 1.33 (Butterfield et al., 1969). By assaying the infectivity of fractions collected following isopycnic

gradient centrifugation, the major peak of infectivity corresponded to a density of 1.30 to 1.31g/ml (Figure 1), comparable with the value reported by Gosting (1977). The minor peaks at 1.34 and 1.40 also were reported by Gosting (1977). No further effort was made to identify these peaks, however, they may represent virions incomplete in maturation. A looser packing of the RNA between the protein subunits in a provirion might allow sufficient cation exchange of cesium ions to cause an increase in buoyant density. Looser packing of RNA in fact was suggested as a reason for the higher density of intact rhinoviruses when compared with the enteroviruses (McGregor et al., 1966). Possibly these higher density components were particles that had eluted from cell surfaces thereby loosing capsid protein. This would serve to increase the RNA to protein ratio thus increasing the density. Several workers demonstrated that one of the virion proteins, VP-4, was lacking in both coxsackie viruses and rhinoviruses that eluted from cells (Crowell and Philipson, 1971; Lonberg-Holm and Korant, 1972). The density of these eluted particles to my knowledge has not been determined, however, one might expect their density to be greater than that of the intact virion.

The size of AEV was reported previously as 23.3 ± 2nm (Krauss and Ueberschar, 1966). These authors, however, failed to mention the standard they used for measurement. Gosting (1977) reported a value of 26 ± 3nm and Butterfield et al. (1969) found AEV to be between 17 and 24nm by filtration. In the present study, UA stained particles were 11% larger than particles stained with PTA. UA-stained virions were shown to be subject to flattening (Serwer, 1977) which could

have caused an apparent increase in size. Wrigley (1969) noted that the orientation of an icosahedral virus must be known for an accurate measurement because 'face to face' and 'corner to corner' diameters differed by 11.5%. All the standard deviations in the reported measurements were within 11.5% of the mean diameters.

The electron microscopic determination of fine structure in small animal viruses is difficult because the viruses do not show the same degree of resolution as other viruses of similar size. For example, poliovirus (Mayor, 1964) and foot-and-mouth disease virus (Breese et al., 1965) do not show nearly the detail as seen in similarly prepared samples of ϕX174 (Markham et al., 1963) or cucumber mosaic virus (Murant, 1965). AEV is no exception because PTA-stained preparations show little surface detail discernable from background grain. The replica method was used to determine fine structure of nucleic acids and ϕX174 (Hall et al., 1959). The method applied to ϕX174 clearly showed 12 knobby structures. By comparison, AEV did not exhibit the 12 protruding capsomers that were proposed by Krauss and Ueberschar (1966). Instead the appearance of three protrusions on several of the sides suggested a 42 or 32 capsomer structure.

The only symmetry observed in spherical viruses was icosahedral or 5:3:2 symmetry, except the polyhedral bacteriophage which had octahedral or 4:3:2 symmetry (Mattern, 1977). Casper and Klug (1962) proposed that the structural units of a capsid were arranged in an icosahedron because it was the structure in which the least distortion of subunit bonds could occur. A minimum of 60 structural units are required to construct an icosahedron, clustered in groups of five

around the 12 verticies. For a larger number of subunits, the triangular faces of the icosahedron are subdivided into smaller triangles to generate icosadeltahedrons. The number of new triangular facets of each original face is termed the triangulation number, "T", Casper and Klug (1962) described the correspondence of each deltahedrons and their triangulation numbers to various icosahedral viruses and to the distribution of capsomers on the virus surface. The triangulation number, T, is described by the equation $T = h^2 + hk + k^2$ where h and k are the coordinates of the vertices of the deltahedron on an isometric grid consisting of equilateral triangles. The number of capsomers is determined by the relationship $C = 10T + 2$. Therefore, AEV must have a triangulation number of 3 (32 capsomers) or 4 (42 capsomers).

A large number of viruses examined to date appear to have the classic hexamer-pentamer clustering of structural units, thus capsomers at the vertices were 5-coordinated (one capsomer surrounded by five others) and all others were 6-coordinated and, therefore, exhibit 6-fold rotational symmetry. A number of viruses, notably members of the picornavirus family, have a number of nonidentical peptide chains in the capsid, complicating the determination of capsomer number. However, Dunker (1974) showed that a capsid containing nonidentical polypeptide chains, resolvable into symmetrically equivalent sets, could give the appearance of 5-chain clusters around the vertices and 6-chain clusters at all the other locations. Thus, using the rotation technique one would expect that 5-coordinated capsomers could be discerned in most viruses. The Markham technique was used to determine 5:3:2 symmetry by a number of investigators (Agrawal, 1966; Breese et

al., 1965; Chambers et al., 1965; Murant, 1965). The results showed that rotation of AEV by this technique enables centers of 5-fold and 6-fold rotational symmetry to be resolved. Therefore, AEV resembled the other isometric viruses which possess 5:3:2 symmetry.

The rotation technique is not without problems. For example, any flaw in the negative that is far enough from the center of rotation and does not overlap with each step will be reproduced with each exposure, creating pseudosymmetry. This possibility can be guarded against, as suggested by Markham et al. (1963), by rotating a number of times that is not an integral periodicity of the actual symmetry. For example, as seen in Figure 6, a 6-step rotation resulted in a blurred print with no reinforcement of symmetry, indicating that the reinforcement of symmetry seen in the corresponding 5-step rotations were true and not the result of pseudosymmetry.

Determination of the exact capsomer number of AEV was difficult because of its small size. Flattening and distortion of the particle may have occurred during the staining process and affected the relative location of capsomers. Fixation prior to staining likely reduced some of the distortion problem. The results suggested a 32 or 42 capsomer structure but, distinguishing between the two was not possible with the methods used.

Previous investigators demonstrated growth of AEV in cell culture but, titers were persistent and low (Mancini and Yates, 1967, 1968a,b; Abe, 1968; Sato et al., 1971; Kodama et al., 1975). Similar results were obtained in the present study with whole embryo cell cultures. Because no inclusions or CPE were observed in the whole embryo

cultures, the cell type(s) infected was not identified. Mancini and Yates (1968) used similar cultures and showed no CPE or inclusions. In addition, infected cultures examined by the fluorescent antibody technique were negative for viral antigen (Sato et al., 1971; Kodama et al., 1975). Cultured cells infected with AEV likely contained insufficient antigen to be detected by FAT. The slow growth of AEV may be because the ideal cell type was not used or, it may be a characteristic of the virus. The initial slow growth of the virus may indicate a very long eclipse period or adaptation of the virus to cell culture.

The type of nucleic acid in some viruses is determined by their sensitivity to RNAse or DNAse, but most viruses are resistant to nuclease (Bernhard and Tourmer, 1962; McAllister et al., 1963). Butterfield et al. (1969) observed a decrease of $10^{0.7}$ EID_{50}/ml in infectivity after RNAse treatment of AEV. However, Gosting (1977) showed no effect of RNAse on the infectivity of purified AEV. Staining of virus-infected cells or purified virus with acridine orange can be used to identify nucleic acid type (Mayor and Diwan, 1961; Mayor and Hill, 1961). Purified AEV stained with acridine orange shows a flame red fluorescence (Matsumoto, 1979). This observation, however, only indicates that the virus contains a single stranded genome because both ss RNA and ss DNA give similar fluorescence. Because AEV fails to produce inclusions in cell culture, other cytochemical staining methods such as Feulgens staining (DeLamater, 1948), are of no use in determining nucleic acid type.

Nucleic acid type was assessed by growing the virus in the presence of inhibitors of DNA replication. When AEV was grown in the presence of BUDR no decrease in virus production was evident after 16 days (Figure 9). Thymidine was included in cell cultures because inhibition by BUDR was reversed by thymidine (Plowright et al., 1966). In the present study thymidine addition actually resulted in a slight increase in virus production possibly because of better cell growth conditions. Bates et al. (1972) showed that BUDR completely depresses virus production of bovine parvovirus 1. Picornaviruses, because of their mode of replication, do not require DNA replication. Therefore, growth in the presence of BUDR suggests that AEV does not contain a DNA genome.

Labelling of virus in cell culture with nucleic acid precursors also was used to determine nucleic acid type. An activity peak was obtained from infected cell culture grown in the presence of ^{3}H-uridine but not in ^{3}H-thymidine-labelled cultures (Figure 10). Prolonged labelling at a high concentration was needed to obtain an activity peak because of the poor virus growth. Although every attempt was made to insure that the peak was viral RNA and not cellular RNA adsorbed to the virion and/or ribonuclear protein of the same density as AEV, these possibilities exist.

Fenner et al. (1974) stated that one of the most reliable methods for determining the nucleic acid type was direct sugar analysis of the viral nucleic acid. This author used the diphenylamine and orcinol methods to show that AEV contained an RNA genome (Table 1). Similar methods were used to analyze other viral nucleic acids (Bachrach et

al., 1963; Salzman and Jori, 1970). The orcinol procedure was not totally specific because deoxyribose reacted with approximately a 25 to 30% intensity of that obtained with an equivalent amount of ribose. Therefore, the RNA detected in the purified AEV samples might be cross reacting DNA; however, this would give an unreasonable value of 60% nucleic acid in the virion. On the other hand, the diphenylamine reaction was specific for deoxyribose but was subject to inhibition by certain proteins especially if they were treated with alkali (Burton, 1969). Because only RNA was detected when the chemical analysis was performed on acid hydrolized products of AEV and phenol-extracted virus, inhibition was not considered as the later preparation contained little or no protein. In addition, the diphenylamine reaction detected DNA in the non-linear gradient purified virus samples which, as indicated above, were likely contaminated with cellular proteins and nucleic acid.

It was essential that the virus be of high purity for accurate chemical analysis. Electron micrographs of isopycnically purified virus were essentially free of impurities. Williams (1954) demonstrated the detection of an impurity present in a concentration of as little as 1% of the virus by electron microscopy, which according to Knight (1975) was the most accurate physical method for assessing purity. A double immunodiffusion test (Matsumoto, 1978) showed only one band between a purified AEV sample and infected chicken serum, again attesting to the purity of the virus for chemical analysis.

Viral nucleic acid was extracted with SDS by heating and centrifuged through a sucrose gradient. The major peak had a sedimentation

coefficient of 32, as determined by the method of McEwen (1967), therefore, assumed to be nucleic acid (Figure 11). The smaller peaks were estimated, from higher to lower sucrose percentage, to be approximately 20S, 12S and 5S. Because the results were from thermally degraded virus, these peaks may represent protein subunits of the virus. It is not known why the peaks were absent in the RNAse treated sample. Possibly, the RNAse was contaminated with proteases. Various subunit antigens of the picornaviruses were reported upon virus degradation. Particles of 5S and 14S (Rueckert et al., 1969), 12S (Talbot and Brown, 1972) and 20S (Philipson et al., 1973) were described. These various subunits can be explained if the picornaviruses were composed of subunits arranged in a 32 capsomer structure (T=3) (Philipson et al., 1973). In a separate experiment with thermal-SDS degraded virus, the 12S peak was absent (Figure 13). Differences between samples might be expected because degradation is difficult to control and as Philipson et al. (1973) indicated, the larger 12S subunit may be composed of three of the smaller 5S subunits.

The percentage of nucleic acid in the virion and the virion molecular weight were calculated assuming that AEV was composed only of protein and nucleic acid, a reasonable assumption because picornaviruses currently are believed to be composed only of RNA and protein. Lipid and carbohydrate content were not assayed in the present study. Although lipid content was not directly determined, AEV was resistent to lipid solvents (Gosting, 1977). Even though it generally is assumed that the small nonenveloped animal viruses contain no carbohydrate, there were reports of its presence (Rueckert, 1965; Lerner and Miranda, 1968). In addition, the hemagglutinin of coxsackie

virus (Tollotson and Lerner, 1966) was inactivated by periodate. Even in view of these observations, there is, to my knowledge, no definitive evidence that carbohydrate is an integral component essential for virus function. Any detectable carbohydrate is likely an adventitious contaminant.

The mean RNA content of AEV was determined to be 27.1%. Reportedly, picornaviruses have RNA contents of: Poliovirus 29% (Schaffer and Frommhagen, 1965); Maus-Elberfield, ME, virus 31.5% (Rueckert and Schafer, 1965); Encephalomyocarditis, EMC, virus 31.7% (Burness, 1970); Mengovirus 21% (Scraba et al., 1967); Rhinovirus type 14, 30% (McGregor, 1969); Foot-and-mouth disease virus, A119, 31.5% (Bachrach et al., 1964). Burness (1970) demonstrated that the orcinol method was reliable for determining virion RNA content. One disadvantage of the method was that an equivalent amount of viral RNA and yeast RNA, used as a standard, may not give the same extinction after the reaction. Burness found a 12% difference in extinction between EMC RNA and yeast RNA but, it cannot be assumed that AEV RNA behaved in a similar manner. Because the determination of the weight of AEV RNA being assayed was not possible, there is a possibility of an error of 12%. A similar relation may exist between extinction with AEV protein and BSA with Lowry's method because a 2% difference in extinction was noted between EMC protein and BSA.

Ultraviolet scans of AEV indicated that the value of 27% RNA was relatively accurate when compared with solutions of various RNA content (Figure 14). While not exceptionally quantitative, comparing UV scans lends further evidence to the reported composition. As

additional evidence for the accuracy of the chemical methods for determining RNA content, TMV analyzed by the above methods gave a percent RNA value essentially the same as reported in the literature.

The extinction coefficient of 74 compares with the reported values for picornaviruses ranging from 76 to 85 (Ruekert, 1971). Because AEV apparently has a slightly lower nucleic acid content than the other picornavirus the extinction coefficient of the virion would be expected to be slightly lower.

The sedimentation coefficient of AEV RNA was 33.6S as determined in 0.1% SDS. Newman et al. (1973) examined the relative sedimentation coefficients of a number of picornviral RNAs in sucrose containing 0.1% SDS. The values were: FMDV, Coxsackie A9, EMC and Equinerhinovirus, 35S; Bovine enterovirus, 33 to 34S; Feline picornavirus, 30-32S; Human rhinovirus, 33S and Vesicular exanthemia, 37S. Poliovirus RNA has a reported sedimentation coefficient of 33S, relative to TMV RNA (Granboulan and Girard, 1969). Few other virus groups had sedimentation values in this range. For the parvoviruses, values of 23 to 27S were reported (Salzman, 1978). The determination of sedimentation coefficients by comparison with other molecules does not necessarily give an accurate value, because the marker molecules may not have the same hydrodynamic properties such as shape and viscosity. In addition, the marker values must be known precisely. The use of SDS in the gradient likely eliminated differences that conformation might cause in determining the sedimentation coefficient.

The molecular weight of AEV nucleic acid was determined to be 2.3 x 10^6 daltons from polyacrylamide gel electrophoresis and 2.4 x 10^6

daltons from the sedimentation studies. The molecular weight of poliovirus was determined by contour length measurement to be 2.6 x 10^6 for the sodium form (Granboulon and Girard, 1969). Similarily, EMC virus RNA was assigned a molecular weight of 2.7 x 10^6 by these authors. However, Fenwick (1968) estimated the size of EMC RNA to be 2.4 x 10^6 daltons. Tannock et al. (1970) found a value of 2.56 x 10^6 daltons. Tannock et al. (1970) found a value of 2.56 x 10^6 daltons for poliovirus RNA by polyacrylamide gel electrophoresis. The parvovirus group, on the other hand, has reported molecular weights of 1.4 to 1.7 x 10^6 daltons for its nucleic acid (Salzman, 1978).

The molecular weight of AEV nucleic acid was based on the molecular weights of TMV, the 28, and 18S ribisomal RNAs. TMV has reported molecular weights of 2.15 x 10^6 daltons (Boedtker, 1960); 1.94 x 10^6 daltons (Boedtker, 1959). The value consistently used in the literature for molecular weight determinations is 2.0 x 10^6 dalton (Tannock et al., 1970; Loening, 1969). There is some variation in the molecular weight values for mammalian 28 and 18S ribisomal RNAs. For 28 and 18S RNAs, reported values are 1.64 and 0.67 (Petermann and Paulovec, 1966), 1.75 and 0.70 (Loening, 1969), 1.65 and 0.65 (Reijnders et al., 1973) million daltons, respectively. To calculate the molecular weight of AEV the average values of 1.68 x 10^6 daltons for 28S and 0.67 x 10^6 daltons for 18S were used.

The determination of particle weights for small viruses is difficult, attested by values reported for picornaviruses ranging from 5.5 x 10^6 to 10.0 x 10^6 (Reuckert, 1971). Because most picornaviruses have similar physical properties, this much difference in their

particle molecular weights is unlikely. The wide variation in results probably represents experimental error because diffusion coefficients and partial specific volumes ($\bar{\nu}$) are difficult to determine accurately. Physical measurement of the diffusion coefficient and partial specific volume of AEV was not attempted because of the small amount of virus available. The partial specific volume was calculated from the determined percent RNA of AEV as suggested by Markham (1967). With EMC, for which $\bar{\nu}$ was experimentally determined, the value calculated by the Markham method was essentially the same as the experimentally determined value (Burness and Clothier, 1970). With the Einstein equation, a diffusion coefficient of 1.52×10^{-7} cm^2/sec was calculated for AEV. Values ranging from 1.44 to 1.9×10^{-7} were reported for picornaviruses (Rueckert, 1971). The radius used in the Einstein equation should be the hydrated radius, because this was not determined, the radius from electron microscopy was used. Burness and Clothier (1970) showed that for EMC, the hydrated radius and that determined by electron microscopy were virtually identical. The virion MW calculated from these data along with the virion sedimentation coefficient of 148S gave a molecular weight value of 7.6×10^6 daltons. A value of 8.5×10^6 was calculated from the nucleic acid molecular weight and its percent in the virion. While differences in these values are considerable, a small error in the percent nucleic acid would make a rather large difference in the molecular weight calculated by the latter method. Both of these values are, however, within the suspected values of the true molecular weight of picornaviruses suggested to be 6.2 to 8.6×10^6 daltons (Rueckert, 1971).

Assuming a value of 320 daltons for an average ribonucleotide (Segel, 1976) the RNA genome of AEV contains about 7,200 nucleotides. Because there is a coding ratio of three nucleotides per amino acid, the genome can code for 2,400 amino acids or, about 288,000 daltons of protein assuming an average molecular weight of 120 daltons for an amino acid (Segel, 1976). The virion has a molecular weight of between 7.6 and 8.5 x 10^6 daltons and an RNA content of 27% thus, AEV would contribute 5.5 to 6.2 x 10^6 daltons to the total virion weight. With a 32 or 42 capsomer model for AEV, each capsomer would weigh approximately 180,000 daltons and 140,000 daltons, respectively. The capsid proteins thus would only account for between 50 and 60% of the genome coding capacity, which is consistent with the synthesis of picornaviral protein. Picornviruses translate their m-RNA into a polyprotein which is cleaved to three primary products. Only one of these, approximately 40 to 50% of the polyprotein, is further processed to structural peptides. The other products are probably involved in intracellular functions such as synthesis of viral RNA (Luria et al., 1978).

The results of this study demonstrated the presence of an RNA genome in AEV directly by chemical analysis and indirectly from labelling experiments and sedimentation studies on nuclease treated samples. Attempts to detect DNA were consistently negative. Although the picornavirus and parvoviruses share many characteristics, the nucleic acid type is the basic criterion for differentiating the two. The known properties of AEV are summarized in Table II. It is concluded that AEV belongs to the family Picornavirdae, in the genus

TABLE II. Biophysical and Biochemical Characteristics of AEV.

Virion	
Density (g/ml)	1.31 - 1.32
Sedimentation coefficient[a]	1.48 ± 3S
Diameter (nm)	27 ± 2
Number of capsomers	32 or 42
Sensitivity to:	
Lipid solvents[b]	-
56 C for 30 min.[c]	+
pH 3.0[c]	-
Trypsin[d]	-
Divalent cation stabilization[c]	+
Extinction coefficient	74
Partial specific volume (ml/g)	0.6885
Diffusion coefficient (cm^2/sec)	1.52×10^{-7}
Molecular weight ($\times 10^6$)	7.6 - 8.5
Nucleic Acid	
Type	ssRNA
Sedimentation coefficient	33.6
Percent in virion	27.1
Molecular weight ($\times 10^6$)	2.3 - 2.4

[a]Gosting (1977)

[b]Gosting (1977); Butterfield et al. (1969); Buelow (1964); Krauss and Ueberschar (1966)

[c]Gosting (1977); Butterfield et al. (1969)

[d]Gosting (1977); Buelow (1964)

Enterovirus. This classification is summarized by the cryptogram: [R/1: 2.3-2.4/27: S/S: V/0].

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