ELECTRON MICROSCOPIC STUDIES OF INFECTIOUS BRONCHITIS VIRUS

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This is to certify that the

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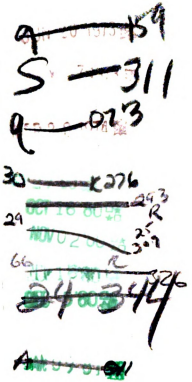

ABSTRACT

ELECTRON MICROSCOPIC STUDIES OF
INFECTIOUS BRONCHITIS VIRUS

by Keyvan Nazerian

Morphological features of infectious bronchitis virus (IBV) were investigated by electron microscopy, using the negative staining technic. The virus in allantoic fluid (AF) and chicken embryo kidney cell (CEKC) cultural medium consisted of homogeneous particles 830-1,000 Å in diameter, with pear-shaped spikes on the surface.

Ether removed the spikes, disrupted the lipoprotein coat, and released the nucleoprotein (NP) helix of the virus. Trypsin also removed the spikes from the surface of the viral particles and disintegrated particles of 330 Å in diameter present in normal allantoic fluid (NAF) and AF containing the virus.

An inhibitor of hemagglutination (HA) activity of IBV was detected in NAF and AF containing the virus. This inhibitor is destroyed by trypsin, ether, and has a surface charge density different from that of the hemagglutinin of the virus and can be selectively removed by diethylaminoethyl-cellulose (DEAE-cellulose) column chromatography.

It is possible that the particles 330 Å in diameter present in AF may be responsible for inhibition of the HA activity of the virus.

Hemagglutinin of IBV as eluted from DEAE-cellulose columns is particulate in nature and has almost the same shape and dimensions of the virus. It adsorbs to the surface of chicken erythrocytes within 30 minutes.

Viral particles adsorb to CEKC within 30 minutes. After 12 hours viral particles were observed in the cytoplasmic matrix and accumulated in the cytoplasmic vesicles within 24 hours. After 42 hours the cytoplasmic vesicles were more numerous and viral particles were also observed in the interstitial areas and on the membrane of infected cells. The virus appeared to release from the cell by budding.

ELECTRON MICROSCOPIC STUDIES OF INFECTIOUS BRONCHITIS VIRUS

By

Keyvan Nazerian

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THIS THESIS IS RESPECTFULLY

DEDICATED

TO

MY FAMILY

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INTRODUCTION

Infectious bronchitis virus has been considered a member of the Myxogroup of viruses. It agglutinates erythrocytes only after treatment with trypsin, ether, or selective elution from DEAE-cellulose columns.

The objectives of this study were to investigate the morphological features of the virus, its hemagglutinin as obtained by trypsin treatment and selective elution from DEAE-cellulose, and the intracellular development of the virus.

LITERATURE REVIEW

Infectious bronchitis virus (IBV)

Infectious bronchitis virus (IBV) is the causative agent of a respiratory disease of chickens. The virus can be propagated in embryonating chicken eggs (Beaudette and Hudson, 1937; Cunningham and Stuart, 1947) and cell culture (Fahey and Crawley, 1956; Cunningham, 1957, 1960). Cytopathic effects (Spring, 1960) and plaques (Cunningham and Spring, 1965) are produced by the virus when grown in chicken embryo kidney cell (CEKC) cultures. The virus contains ribonucleic acid (RNA) and is ether sensitive (Akers, 1963).

Normally the virus does not adsorb to the surface of erythrocytes. Treatment of the virus with trypsin (Corbo and Cunningham, 1957), ether or selective elution of the virus by diethylaminoethyl (DEAE)-cellulose column chromatography (Biswal, 1965) induces the hemagglutinating activity of the virus. Trypsin modified virus reduces the electrophoretic mobility of the erythrocytes (Biswal, 1963). The virus forms syncytia in CEKC cultures after 8 hours post-inoculation. Viral replication and syncytial formation are inhibited by DL-fluoro-phenylalanine but not by aminopterin (Akers, 1963). The virus disintegrates into three different

antigens after heating at 100 C for 30 minutes, treatment with ether or sodium dodecyl sulfate. These antigens are also present as soluble antigens in the allantoic fluid containing the virus. Cesium chloride density gradient centrifugation and DEAE-cellulose column chromatography have been successfully used to separate these three antigens (Tevethia, 1964). The virus is present in the allantoic fluid in two phases, the thermolabile D phase and the thermostable O phase (Singh, 1960). The virus has optimal stability at pH 7.8 and its isoelectric point is at pH 4.05 (Cunningham and Stuart, 1947). The virus has a sedimentation coefficient of 334 S and specific gravity of 1.24 and 1.19 as determined by cesium chloride density gradient and sucrose gradient centrifugation, respectively (Ellis, 1965).

Fluorescent antibody studies indicate the presence of specific antigens in the cytoplasm of CEKC 2½ hours postinoculation (Stultz, 1962). Mohanty (1964), however, reported the presence of specific antigen in the nucleus of CEKC 7 hours postinoculation and in the cytoplasm only after 36 hours.

The virus particle has a diameter of 600 Å to 1,200 Å (Reagan and Bruchner, 1952; Reagan et al., 1948; Buthala, 1956; Nazerian, 1960; and Berry et al., 1964). In the chorioallantoic membrane (CAM), elementary bodies as large as 2,000 Å in diameter are present in the cytoplasm (Domermuth, 1957).

Electron microscopy

The particulate nature of viruses was first confirmed by electron microscopy. Viruses like other hydrated biological materials give a very poor contrast when examined directly in the electron microscope. The shadowcasting technic greatly assisted the morphological studies of viruses. Viral particles deposited on the supporting film are shadowed with an electron dense heavy metal at an appropriate angle. Such preparations when examined in the electron microscope reveal the shape and dimensions of the virus with much higher contrast. Using a known concentration of polystyrene balls shadowcasting has been used for particle counting by the droplet technic. By shadowing viral particles from two directions 30 to 45 degrees apart it is possible to determine the symmetry of some of the viruses.

Not much was known of the detailed structure of viruses and their inner components until the negative staining technic was developed by Brenner and Horne (1959). Several chemicals have been used as stains but 1 to 2 per cent solution of phosphotungstic acid adjusted to pH 7.0 with potassium or sodium hydroxide is the one most widely used. If spread evenly phosphotungstic acid forms an electron dense film around the virus particles and penetrates between their subunits. The electron beam passing through the particles forms a high contrast image as viewed on the fluorescent screen of the microscope. Many details of viral

structure have been studied through the application of this technic. "Positive staining" with uranyl acetate has been developed by Smith and Melnick (1962) to differentiate between the DNA viruses and RNA viruses. Viruses containing DNA stain very heavily with uranyl acetate in contrast to the RNA viruses.

Electron microscopy has also been used to study the intracellular development of viruses. The development of suitable fixatives, embedding material, and the ultramicrotome have facilitated this study. Osmium tetroxide fixatives developed by Palade or Dalton are commonly used. Methacrylate was the first polymer used as an embedding material. Epoxy resin (Fink, 1960; Luft, 1961) and Vestopal W (Ryter and Kellenberger, 1958) were developed later and proved to cause less damage to the cells than methacrylate.

Thin sections of cells infected with viruses have been extensively studied for localization of inclusion bodies and intracellular replication of viruses. Ferretin-conjugated specific gamma globulin has been used to label viral particles (Singer, 1959; Rifkind, 1960; Sri Ram et al., 1963; Morgan et al., 1962) and their antigens. Using this technic it is possible to differentiate viral particles from otherwise indistinguishable particles of cellular nature.

Autoradiography also has been applied to determine the site of synthesis of viral macromolecules. Supplying the cells with radioactive amino acids, purines or pyrimidines

viral macromolecules will be labeled with radioactive material. When sections of these cells are exposed to photographic emulsion and subsequently developed silver metal will form a deposit on the radioactive labeled viral macromolecules. Such a deposit of metal will localize the site of synthesis of viral protein or nucleic acid depending on the type of radioactive material incorporated.

Viral structure

Viral particles are composed of a species of nucleic acid, DNA or RNA, and a protein coat surrounding the nucleic acid. Upon entering a susceptible cell the nucleic acid replicates and also directs the synthesis of viral protein. These two components then form a complete viral particle.

X-ray diffraction studies on some of the small viruses (Watson, 1956; Casper, 1956) suggested the presence of symmetry in their structure. Crick and Watson (1956, 1957) pointed out that the small amount of nucleic acid in the chemical composition of viruses is not sufficient to direct the synthesis of a complete molecule as protein coat. It was, therefore, suggested that this mucleic acid directs the synthesis of protein subunits that later assemble together and form the complete protein coat of the virus.

This was fully demonstrated when the negative staining technic was developed and the first electron micrographs of adenovirus type 5 were taken by Horne et al. (1959).

The virus is in the form of an icosahedron with five fold, three fold, and two fold axes of symmetry with six morphological subunits on each edge of the icosahedron. The terms "capsid" and "capsomere" were proposed by Lwoff et al. (1959) to represent the viral protein coat and its subunits, respectively. These capsomeres are only the morphological subunits but not the chemical subunits of the protein coat. Nixon and Woods (1960) have shown the chemical subunits of the protein coat of tobacco mosaic virus (TMV) to be different from capsomeres. The capsomeres, furthermore, are different from subunits in that they are all symmetrical but some are on the edges, some are facets, and some are on the corners of the icosahedron. Capsomeres on the corner are surrounded by five other capsomeres while others are surrounded by six.

This type of symmetry was then observed in many other viruses, having identical or different numbers of capsomeres on their edges. Wildy et al. (1960) found that herpes simplex virus also has icosahedral symmetry but the capsomeres are hollow and elongated. It is not very easy to calculate the number of capsomeres and their arrangement in all viruses having icosahedral symmetry. The size of the virus, arrangement of the capsomeres, and the number of capsomeres on the edge are some of the difficulties arising from the species of the virus studied, while formation of two different images of the upper and lower surfaces of the virus, and the axis of symmetry viewed are among the technical problems arising

controversy as to the exact morphology of some of the small viruses like polyoma virus (Wildy et al., 1960; Williams et al., 1961; Melnick, 1962).

Among the viruses having icosahedral symmetry are adeno (Horne et al., 1959), herpes simplex (Wildy et al., 1960), reo (Rhim et al., 1961), polyoma (Wildy et al., 1960), wart (Williams et al., 1961), polio (Horne and Nagington, 1959), and many others.

Some other viruses have helical symmetry. Chemical analysis and X-ray diffraction studies on particles of TMV have long established the symmetry in the structure of this virus. The protein coat is in the form of a helix surrounding the nucleic acid. The subunits of this protein coat are able to reassemble after being separated from the nucleic acid and to form rods morphologically identical with the intact virus (Horne, 1963). This nucleoprotein (NP) helix is rigid and naked in TMV while in other RNA viruses like the myxoviruses is not rigid and is folded and surrounded by an envelope having projections on the surface (Waterson, 1962).

Myxoviruses are a group of RNA viruses, having helical symmetry. They are sensitive to ether, and generally agglutinate erythrocytes by attachment to their mucoprotein receptors (neuraminic acid) (Wilner, 1964). Horne et al. (1960) divide these viruses into two subgroups: (1) influenza and fowl plague; (2) Newcastle disease virus (NDV) and mumps. Influenza and fowl plague are morphologically identical.

They are about 800-1,000 Å in diameter with an envelope 70-100 Å thick. Surface projections are 80-100 Å long and 70-100 Å apart. The internal NP helix is seldom seen in normal preparations of the virus. After ether treatment, however, the NP helix is released and can be separated from the hemagglutinin particles (rosettes) of the virus by centrifugation (Rott and Schafer, 1961; Horne et al., 1960; Hoyle et al., 1961, 1962; Waterson, 1962). The nucleoprotein helix is 90-100 Å in diameter. Hemagglutinin particles are spherical with spikes on the surface. These particles contain hemagglutinin, neuraminidase, and the antigenic and lytic properties of the virus.

Mumps and NDV are considerably larger than influenza viruses. The NP helix is spontaneously released and can be detected in normal preparations of the virus. The helix is 150-170 Å in diameter (Horne, 1963; Horne and Wildy, 1963), and has a central axial hole of 50 Å in diameter, and it resembles very much the helix of TMV in these features. There are fewer projections on the surface than with influenza and fowl plague viruses. High resolution micrographs of Sendai virus, belonging to this subgroup, indicate the presence of more than one helical strand in the NP helix of the virus (Waterson, 1962).

Incomplete hemagglutinating influencza particles released from cells infected with an abnormally high dose of virus (von Magnus, 1951; Henle et al., 1955) are void of an

NP internal helix (Rott and Schafer, 1961). Incomplete hemagglutinating particles of NDV, however, are released normally from the infected cells along with the infective particles. These particles are also void of an NP helix.

Hemagglutination and its inhibitors

Hirst (1941) and McClelland and Hare (1941) independently reported that influenza virus was able to agglutinate chicken erythrocytes. This capacity of hemagglutination (HA) has been reported for many other viruses. Many problems of virus-cell interactions have been investigated by means of this phenomenon.

Not all viruses possess the ability to agglutinate erythrocytes and there are differences in the mode of action of the viruses which possess this activity. Based on their mode of action on erythrocytes, viruses can be classified into two main groups: (1) those which directly agglutinate cells, and (2) those which indirectly cause agglutination of the cells. The first group is divided into three subdivisions: (a) myxoviruses in which adsorption of the virus to the cell, neuraminidase activity of the virus resulting in receptor destruction and release of N-acetyl neuraminic acid (NANA), and finally elution of the virus from the cell, are the three successive steps involved in the virus-erythrocyte interaction; (b) some viruses such as those of vaccinia, variola, ectromelia, and meningopneumonitis in which the hemagglutinin is separable from

the virus by centrifugation and filtration (Lennett, 1959; Anderson, 1959) and DEAE-cellulose column chromatography, and is in the form of a soluble lipoid antigen; and (c) some of the arthropod-borne viruses such as Japanese B encephalitis in which the virus particle is believed to be the hemagglutinin. No neuraminidase is present and there is no receptor destruction or elution of the virus (Anderson, 1959).

Hemagglutination by the second group for example, herpes simplex, can be demonstrated by an indirect method (Lennett, 1959). If sheep erythrocytes are treated with tannic acid and the virus is adsorbed to them, the addition of specific antiserum will cause the cells to agglutinate (Scott et al., 1957). This is the opposite of the action of the first group where hemagglutination is inhibited by specific antiserum. An indirect HA test has been reported for two strains of IBV with tannic acid modified horse erythrocytes (Brown et al., 1962).

Treatment of myxoviruses with ether has resulted in the rupture of the virus and release of the hemagglutinin and S antigen (Hoyle, 1950; 1952; Paucker et al., 1959). The S antigen is the internal helix of viral NP and is associated with infectivity of the virus. The hemagglutinin entity bears the surface projections of the virus and has the hemagglutinating property, neuraminidase activity, lytic activity, and antigenicity of the virus. These properties are all present in the "rosette" shaped hemagglutinin

of NDV, while lytic activity of HA particles of influenza and fowl plague viruses is negligible. It is believed that the hemagglutinin of myxoviruses is a protein molecule different from neuraminidase though both are stereospecific for sialic acid groupings on the surface of erythrocytes. Differential treatment of influenza virus with heat or periodate inactivates the neuraminidase while the hemagglutinating activity of the virus is preserved (Buzzel and Hanig, 1958). This modified virus, termed indicator virus, is able to agglutinate erythrocytes but unable to elute from their surface.

Hemagglutination is inhibited by specific antiserum, nonspecific factors in normal serum (Chu inhibitor) and a variety of mucoid substances of biological origin such as human urine (Tamm and Horsfall, 1952), bovine serum (Curtain and Pye, 1955), egg-white inhibitor (Gottschalk and Lind, 1959), and allantoic fluid (Knight, 1944). Neuraminidase, first isolated from Vibrio cholerae also is able to inhibit the HA activity.

The mucoproteins of HA can completely prevent the activity of the indicator virus. Inhibitors of HA are believed to be mucoprotein or mucolipid in nature, having neuraminic acid groupings as prosthetic groups linked to a protein molecule. They are able to compete with the neuraminic acid of the cell for the virus. The degree of inhibition depends on the concentration of inhibitor, resemblance of inhibitor molecules to the cell receptor, and

the presence of neuraminidase in the virus. Indicator virus and viruses lacking neuraminidase or with little enzyme are readily affected by the inhibitor, while viruses with neuraminidase activity are able to destroy the inhibitor molecule and still be able to attach to the erythrocytes.

Inhibotors of HA isolated from human urine are bundles of glycoprotein 10, 20, and 40 Å wide. These bundles attach to the surface of projections of influenza virus strain A/Singapore and neuraminidase molecules extracted from Vibrio cholerae (Bayer, 1964). Plate-like structures 100-150 Å thick and variable in size have been isolated from bovine serum by Ackermann (1964) and attach to periodate treated influenza virus strain A/57 blocking its HA activity.

Allantoic inhibitor of HA is destroyed by trypsin and periodate (Buzzel and Hanig, 1958). Most but not all of its activity is lost at 100 C for one hour indicating that the inhibitor molecule is coated with proteinaceous materials. Some mucolipid substances inhibitory to the HA activity of viruses have been isolated by Rosenberg et al. (1956), and Bogoch (1957).

These inhibitors of HA are all susceptible to neuraminidase and proteolytic enzymes indicating the presence of ketosidically linked sialic acid as a prosthetic group with a protein molecule in their structure (Gottschalk, 1960).

MATERIALS AND METHODS

Virus

Infectious bronchitis virus (IBV) strains Massachusetts, IBV-41-5; egg adapted Beaudette, IBV-42, and tissue culture adapted Beaudette, IBV-42-113C from the North Central Repository at Michigan State University were used. Each strain is designated with two numbers: the first is the code number of the virus and the second is the number of passages in egg or tissue culture following initial isolation from infected chickens. Strains 41 and 42 were propagated in embryonating chicken eggs while IBV-42-113C was grown on chicken embryo kidney cell (CEKC) cultures.

Preparation of chicken embryo kidney cell (CEKC) cultures

The kidneys from 17 day old chicken embryos were removed aseptically and washed thoroughly in Hanks balanced salt solution (BSS). The kidneys were then minced into pieces of about 1 mm^3 and rinsed twice in BSS. The minced pieces were then incubated for 90 minutes in 0.25 per cent trypsin (Difco, 1:250) in BSS, pH 8.0 to 8.2, in a 500 ml fluted Erlenmeyer flask containing a "Teflon" covered magnet. Ten ml of trypsin solution was used for each pair of kidneys and the flask was

placed over a "Magnestir" throughout the incubation period in order to accelerate the action of trypsin. The cell suspension was then filtered through two layers of gauze. The cells were then separated from the suspension by centrifugation at 437 x G for five minutes and washed twice by suspension in BSS and centrifugation at 437 x G for five minutes. The packed cells were resuspended 1:200 in Eagle's basal medium 199 supplemented with five ml heat inactivated newborn calf serum, one ml of amino acid mixture, one ml of vitamin mixture, one ml of glutamine, 100,000 units penicillin, and 0.1 mg dihydrostreptomycin per 100 ml of the media. Falcon plastic dishes, 60 x 15 mm, were seeded with 4 ml of the kidney cell suspension in medium 199. The cultures were incubated at 37 C in an atmosphere of 8 per cent CO_2 and 80 to 85 per cent humidity. Monolayers of CEKC were formed and 48 hours after seeding the cultures were suitable for infection with the virus.

Propagation of the virus

Strains IBV-41-5, and IBV-42 were propagated in the allantoic cavity of 9 to 11 day old embryonating chicken eggs using 10^3 EID_{50} in 0.1 ml of viral dilution as the inoculum. Embryos were infected via the allantoic cavity. Living embryos after 48 hours postinoculation in the case of IBV-41 and 24 hours in the case of IBV-42 were chilled overnight at 4 C. The allantoic fluid was harvested aseptically and

stored at -72 C in 20 ml screw cap vials. IBV-42-113C was grown on CEKC monolayers (Cunningham, 1963). The cultural medium from 48 hour monolayers of CEKC cultures was decanted. The monolayers were washed thoroughly with BSS and were inoculated with 10^4 PFU in 0.5 ml of viral dilution in BSS containing 5 per cent newborn calf serum. The cultures were incubated at 37 C for 90 minutes for adsorption of the virus to the cells. The inoculum was decanted and four ml of fresh medium 199 was added to each plate. The cultures were incubated for another 48 hours at 37 C in an atmosphere of 8 per cent CO_2 and 80 to 85 per cent humidity. Cultural medium was removed and centrifuged at 400 x G to remove the cellular debris and the virus suspension was stored at -72 C in 10 ml screw cap vials.

Normal allantoic fluid

Allantoic fluid from non-infected 11 day old chicken embryos was harvested aseptically and stored at -72 C in 10 ml screw cap vials.

Assay of viral infectivity

Strains IBV-41 and IBV-42 were assayed for infectivity using 9-11 day old embryonating chicken eggs. Serial ten fold dilutions of the virus were made in nutrient broth. Five embryos were inoculated with 0.1 ml per embryo per dilution via the allantoic cavity. The infectivity titer of

the virus was calculated according to the method of Reed and Muench (1938) expressed in EID_{50}. Mortality, curling, and stunting of the embryo, and urates in the kidneys were considered the criteria of infection.

The tissue culture adapted strain 42 of the virus was assayed for infectivity by plaque assay technic on CEKC cultures (Cunningham and Spring, 1965), the titer being expressed in plaque forming units (PFU). Serial two fold dilutions of the virus were used to determine the titer of the virus. Two monolayer cultures were inoculated with 0.5 ml of each dilution and were incubated at 37 C for 90 minutes. The inocula were decanted completely and replaced with 4 ml of cultural medium 199 containing 0.9 per cent Special-Agar-Noble (Difco). Agar overlay cultures were incubated at 37 C in an atmosphere of 8 per cent CO_2 and 80-85 per cent humidity. At the end of the 72 hours postinoculation the monolayers were stained with 0.5 ml of 0.1 per cent Difco-Bacto neutral red in phosphate buffered saline (PBS), pH 7.0. Cultures were incubated at 37 C for 30 minutes and 4 C for 90 minutes. Infectivity titer was calculated based on number of plaques using the enumerative dose response and is expressed in PFU per ml.

Erythrocytes

Erythrocytes were obtained by cardiac puncture from Single Comb White Leghorns, using two ml of a two per cent

solution of sodium citrate as the anticoagulant for each 10 ml of blood. The cells were washed by centrifugation with three changes of saline solution and were finally removed from the suspension by centrifugation at 500 x G for 15 minutes. A 0.5 per cent cell suspension in saline solution was used for hemagglutination (HA) tests.

Induction of hemagglutination by trypsin

Undiluted IBV-41 infected allantoic fluid, two parts, was added to one part of a one per cent suspension of Trypsin (Difco 1:250) and the mixture was incubated at 56 C for 30 minutes. Egg-white trypsin inhibitor, one part of a ten per cent suspension, was then added to the mixture to stop the action of trypsin (Muldoon, 1960). Serial two fold dilutions of the modified virus were prepared in saline solution. In each of 12 x 75 mm tubes, 0.25 ml of Bacto HA buffer, pH 7.3 to 7.4 and 0.25 ml of the corresponding virus dilution were mixed. To this suspension was added 0.25 ml of a 0.5 per cent suspension of chicken erythrocytes in saline solution. The tubes were shaken for 10 seconds and incubated at room temperature. The reaction was recorded according to the pattern method. The HA titer was the reciprocal of the highest dilution of the virus producing complete hemagglutination.

Diethylaminoethyl (DEAE)-cellulose column chromatography

Anion exchange DEAE-cellulose (Cellex-D 0.78 g equi., CalBioChem) was used as the adsorbant. The DEAE-cellulose was suspended in 0.01 M phosphate buffer, pH 7.4 and was allowed to sediment. Fine particles still in the suspension were decanted. This was repeated several times until no fine particles remained in the suspension. Glass cylinders 10 mm x 200 mm, fitted with a fritted disc were used for making the columns which were packed at 4 C and washed with large volumes of the initial buffer. The virus preparations were thawed at room temperature, centrifuged to remove cellular debris, and then dialyzed against several changes of 0.05 M NaCl in 0.01 M phosphate buffer, pH 7.4. Five ml of the dialysate were added to the column and allowed to adsorb to the column. Phosphate buffer, 0.01 M, pH 7.4, with increasing molarities of NaCl from 0.05 to 1.0 M was passed through the column in order to selectively elute different components of the virus. Fractions were collected at 4 C in five ml portions, using a photosensitive volumetric fraction collector. Each fraction was then analyzed for absorbancy at 2,600 and 2,800 Å in a Beckman DB spectrophotometer, assayed for HA activity, and infectivity. Fractions with HA activity were further used for electron microscopy.

Ultracentrifugation

A model L preparative ultracentrifuge (Beckman Instruments Inc., Spinco Division) was used for purification and concentration of the virus. Infected allantoic fluid, trypsin modified virus, tissue culture media containing the virus, and hemagglutinating fractions obtained after DEAE-cellulose chromatography were separately subjected to centrifugation. All materials were first centrifuged at 10,000 x G to remove cellular materials. The virus was then sedimented at an average centrifugal force of 74,000 x G at 4 C for 60 minutes, using 10 ml lusteroid tubes in a fixed-angle type 50 rotor. The pellets were suspended in PBS, pH 7.4. Duplicate pellets were suspended in one per cent ammonium acetate for electron microscopy. Both the supernatant fluid and the sediment were assayed for infectivity, and HA activity for appropriate experiments.

Cesium chloride density gradient centrifugation

For density gradient centrifugation (Meselson et al., 1957) a SW 39 swinging bucket rotor was used in the model L centrifuge. To 4.7 ml of a 2.5 M cesium chloride in phosphate buffer, pH 7.4 in 5 ml lusteroid tubes, was added 0.5 ml of the virus preparation. Centrifugation was continued for 24 hours at an average centrifugal force of 115,000 x G at 4 C. The rotor was allowed to decelerate

without the use of the brake. Fractions of two drops each were collected by piercing the bottom of the tube with a 26 gauge hypodermic needle. The fractions were then analyzed for specific gravity, ultra-violet absorbancy at 2,600 and 2,800 Å wave lengths and assayed for infectivity and HA activity.

Ether treatment

The tissue culture adapted IBV-42-113C was first purified and concentrated by two cycles of centrifugation. The titer was 10^9 PFU or greater per ml. One part of this preparation was incubated with one part of diethyl-ether at 4 C for 6 hours. The mixture was kept over a "Magnestir" in an Erlenmeyer flask containing a "Teflon" covered magnet. At the end of the incubation period the mixture was centrifuged and ether was removed by a capillary pipette. The residual ether was removed by passing dry nitrogen through the preparation which was then used for electron microscopy.

Electron microscopy

Negative staining by the pseudoreplica technic

The method used was slightly different from that of Rhim et al., (1961), and Smith and Melnick (1962). Special-Agar-Noble 2.5 per cent, in distilled water was poured into glass petri dishes to form a layer of about 5 mm. Using a

scalpel, small blocks of 10 x 10 mm were cut and placed on a clean glass slide. A small drop of the purified virus preparation was spread over the surface of the block. As the liquid penetrated the agar the viral particles remained on the surface of the block. A few drops of a one per cent nitrocellulose solution in amyl acetate were spread over the surface of the block and filter paper was pressed against the edges to blot the excess liquid. Each edge of the block was cut with a scalpel and the pseudoreplica on the surface of the agar was floated off onto a 1.5 per cent solution of silicotungstic acid, pH 7.0 in a 50 ml beaker containing 2-4 drops of Neo-Chem (one drop of Neo-Chem in 200 ml distilled water) and 7 drops of 0.025 per cent solution of calcium chloride. The pseudoreplica thus negatively stained was removed from the silicotungstic acid solution, mounted on No. 2200 Effa grids, and allowed to dry. All preparations were then coated with a thin layer of carbon in a vacuum evaporator and examined in a Model EMU 3E RCA electron microscope. All micrographs were taken at 12,000 instrumental magnification.

Preparation of agglutinated erythrocytes for electron microscopy

Chicken erythrocytes were treated with the hemagglutinating fraction obtained after DEAE-cellulose column chromatography as in the standard HA test. After incubation for 30 minutes at room temperature, three parts of distilled water were added to the mixture and the container was shaken

for 30 seconds to partially hemolyze the erythrocytes. The hemolyzed cells were then fixed in 0.5 per cent osmium tetroxide in Palade's buffer (1952) for 10 minutes, removed from the suspension by centrifugation, washed and resuspended in distilled water. Small drops of this suspension were placed on No. 2200 Effa grids previously coated with Formvar (0.25 per cent in ethylene dichloride) and allowed to dry at room temperature. Specimens were shadowed with chromium at an angle of approximately 30 degrees in a vacuum evaporator. These specimens were examined in a Philips 100-B electron microscope. All micrographs were taken at 3,500 instrumental magnification.

Preparation of CEKC cultures infected with IBV-42-113C for electron microscopy

Cultures of CEKC infected with IBV-42-113C for 1, 12, 24, and 42 hours as well as non-infected controls were used. The monolayers were chilled at 4 C before fixation. The cultural medium was decanted and replaced with one ml of a one per cent solution of osmium tetroxide (Merck) in Dalton's buffer (1955) containing one per cent potassium dichromate and 0.85 per cent NaCl, pH 7.2. After the monolayers of cells were fixed for 60 minutes at 4 C they were then washed in distilled water for five minutes, and removed from the plastic petri dish with a scalpel. Dehydration of the cells in ethyl alcohol and acetone was done according to the method of Ryter and Kellenberger (1958):

Dehydrating mixture	Dehydration period
50% ethyl alcohol	10 minutes
70% ethyl alcohol	15 minutes
70% ethyl alcohol	15 minutes
80% ethyl alcohol	15 minutes
95% ethyl alcohol	15 minutes
95% ethyl alcohol	15 minutes
Absolute ethyl alcohol	7 minutes
Absolute ethyl alcohol	7 minutes
Absolute alcohol and dry acetone equal parts	5 minutes
100% acetone	5 minutes

The cells were infiltrated in four different mixtures of Vestopal W (Martin Jeager, Specialites pour laboratoires, Geneva, Switzerland) and acetone each for 60 minutes:

Mixture 1: One part Vestopal and three parts acetone.

Mixture 2: One part Vestopal and one part acetone.

Mixture 3: Three parts Vestopal and one part acetone.

Mixture 4: Vestopal containing one per cent tertiary butyl perbenzoate, one per cent cobalt naphthenate as initiator and activator, respectively. The monolayers were then packed by centrifugation and placed in the bottom of No. 0 rectal gelatin capsules. Vestopal containing initiator and activator were added to fill the capsules. Polymerization of Vestopal was accomplished at 60 C for 24 hours in a dry oven.

Preparation of thin sections of CEKC monolayers and electron microscopy of the sections

The gelatin was removed from the hardened Vestopal blocks by submerging the capsule in warm water. Using a razor blade small pyramids were trimmed at the top of each block to give a sectioning area of about 0.1 mm square. Sections of about 600 Å in thickness were cut by the LKB Ultratome using a glass knife. Sections were examined in a Model EMU 3G RCA electron microscope. Micrographs were taken at 5,000 instrumental magnification.

RESULTS

Virus

Ninety one per cent of the infectious IBV-42-113C particles were present in the sediment following the first cycle of centrifugation at an average centrifugal force of 74,000 x G for 60 minutes. Duplicate pellets suspended in one per cent ammonium acetate and PBS had a titer of 5.2×10^6, and 5.4×10^6 PFU per ml, while the titer of the supernatant fluid was 5.4×10^5 PFU per ml. Sediment suspended in one per cent ammonium acetate was recentrifuged and resuspended in ammonium acetate for electron microscopy. This sample had a titer of 1.2×10^9 PFU per ml. The viral particles were homogeneous in shape with the size ranging from 750 to 950 Å in diameter and pear shaped spikes on the surface of the viral particles (Figure 1). A rather homogeneous population of infective particles corresponding to a specific gravity of 1.29 was obtained after cesium chloride density gradient centrifugation (Figure 2 and Table 1).

Treatment of the partially purified IBV-42-113C with ether resulted in removal of the spikes, disruption of the lipoprotein coat of the virus, and release of the helical NP materials (Figures 3 and 4).

Table 1: Cesium chloride density gradient centrifugation of partially purified IBV-42-113C

Fraction No.	Specific gravity	Infectivity (PFU/ml)
1	1.480	0
2	1.428	0
3	1.412	0
4	1.392	0
5	1.388	0
6	1.368	0
7	1.352	0
8	1.348	0
9	1.340	0
10	1.332	0
11	1.328	1.3×10^3
12	1.324	4.5×10^4
13	1.300	1.1×10^5
14	1.280	7.5×10^4
15	1.288	5.3×10^3
16	1.228	6.7×10^3
17	1.252	4.0×10^3
18	1.204	3.3×10^3
19	1.208	2.6×10^3
20	1.212	1.1×10^3
21	1.176	4.4×10^2
22	1.188	0
23	1.128	1.2×10^3

In the embryo passaged strain 41-5 purified by centrifugation (Figure 5) there were two types of particles: One type was morphologically identified as virus particles similar to the strain 42-113C (Figures 19 and 20), and the other type identical with 330 Å particles normally present in allantoic fluid (Figure 6).

Trypsin modified virus

Trypsin modified IBV-41-5 sedimented at 74,000 x G for 60 minutes formed a pellet which was very loose in contrast to the pellet from non-modified virus at the same centrifugal force. When 136,000 x G was applied for 120 minutes without the use of the brake the pellet was firm. The sediments and supernatant fluids were assayed for HA activity. Results are recorded in Table 2.

Table 2: Centrifugation of trypsin modified IBV-41-5

Centrifugation conditions	Ha titer before centrifugation	HA titer in the supernatant	HA titer in the pellet
74,000 x G for one hour	2048	1024	8
136,000 x G for two hours	2048	256	1024

After trypsin modification spikes were removed from the surface of the virus, and particles with 330 Å diameters normally present in NAF and AF containing the virus were disintegrated (Figure 7).

The observation that trypsin destroyed some particles normally present with the virus in allantoic fluid suggested that these particles might be inhibitors of direct HA by the virus. Four standard HA tests were performed using,

(1) HA buffer containing 10 per cent NAF, (2) HA buffer containing 10 per cent AF with IBV-41, ($_3$) HA buffer containing 10 per cent trypsin modified NAF, and (4) normal HA buffer as control. Results are recorded in Table 3.

Table 3: Hemagglutination inhibition of IBV-41 by NAF and AF containing the virus.

Virus	HA buffer	HA Titer
Trypsin-modified virus	Normal HA buffer	128
Trypsin-modified virus	10% NAF in HA buffer	0
Trypsin-modified virus	10% AF containing IBV-41 in HA buffer	0
Trypsin-modified virus	10% trypsin-modified NAF in HA buffer	128

Data in Table 3 indicate that NAF as well as AF containing IBV-41 inhibits the HA activity of the modified virus. Trypsin modified NAF, however, had no inhibitory effect. This indicates the presence of an HA inhibitor in NAF and AF containing the virus.

Hemagglutinin fraction of IBV-41 obtained after DEAE-cellulose column chromatography

The fractions eluted from DEAE-cellulose at 0.1 to 0.15 M NaCl has HA activity (Biswal, 1965). This fraction consisted of spherical particles 800 Å in diameter (Figure 8) which adsorb to the surface of chicken erythrocytes within

30 minutes (Figure 9) forming bridges between erythrocytes and causing agglutination (Figure 10). Hemagglutinin particles are also able to attach to the stroma isolated from hemolyzed cells (Figure 11).

Data in Table 4 show the inhibitory effect of NAF and AF containing IBV-41 confirming the presence of an HA inhibitor in AF sensitive to trypsin.

Table 4: Hemagglutination inhibition of IBV-41 by NAF and AF containing the virus.

Virus	HA buffer used	HA titer
Hemagglutinin*	Normal HA buffer	65
Hemagglutinin*	10% NAF in HA buffer	0
Hemagglutinin*	10% AF containing IBV-41 in HA buffer	0
Hemagglutinin*	10% trypsin modified NAF in HA buffer	64

*As eluted from DEAE-cellulose column.

CEKC cultures infected with IBV-42-113C

Viral particles were adsorbed to the membrane of CEKC within 30 minutes (Figure 12). After 12 hours viral particles, 900 Å in diameter, were observed in the cytoplasmic matrix of the cells (Figure 14). Cytoplasmic vesicles containing viral particles were present 24 hours postinoculation (Figure 15) and were more numerous at 48 hours (Figure 16).

At this time viral particles were accumulated in the interstitial areas and were also present on the surface of the membrane. The nuclear membrane was ruptured in some cells releasing virus-like particles into the cytoplasm (Figure 17). The virus appears to be released from the cell by budding (Figure 18).

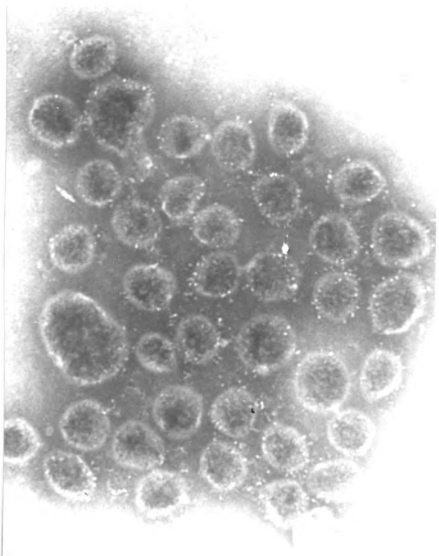

Figure 1: Electron micrograph of IBV-42-113C. Negatively stained with silicotungstic acid (STA). X 120,000

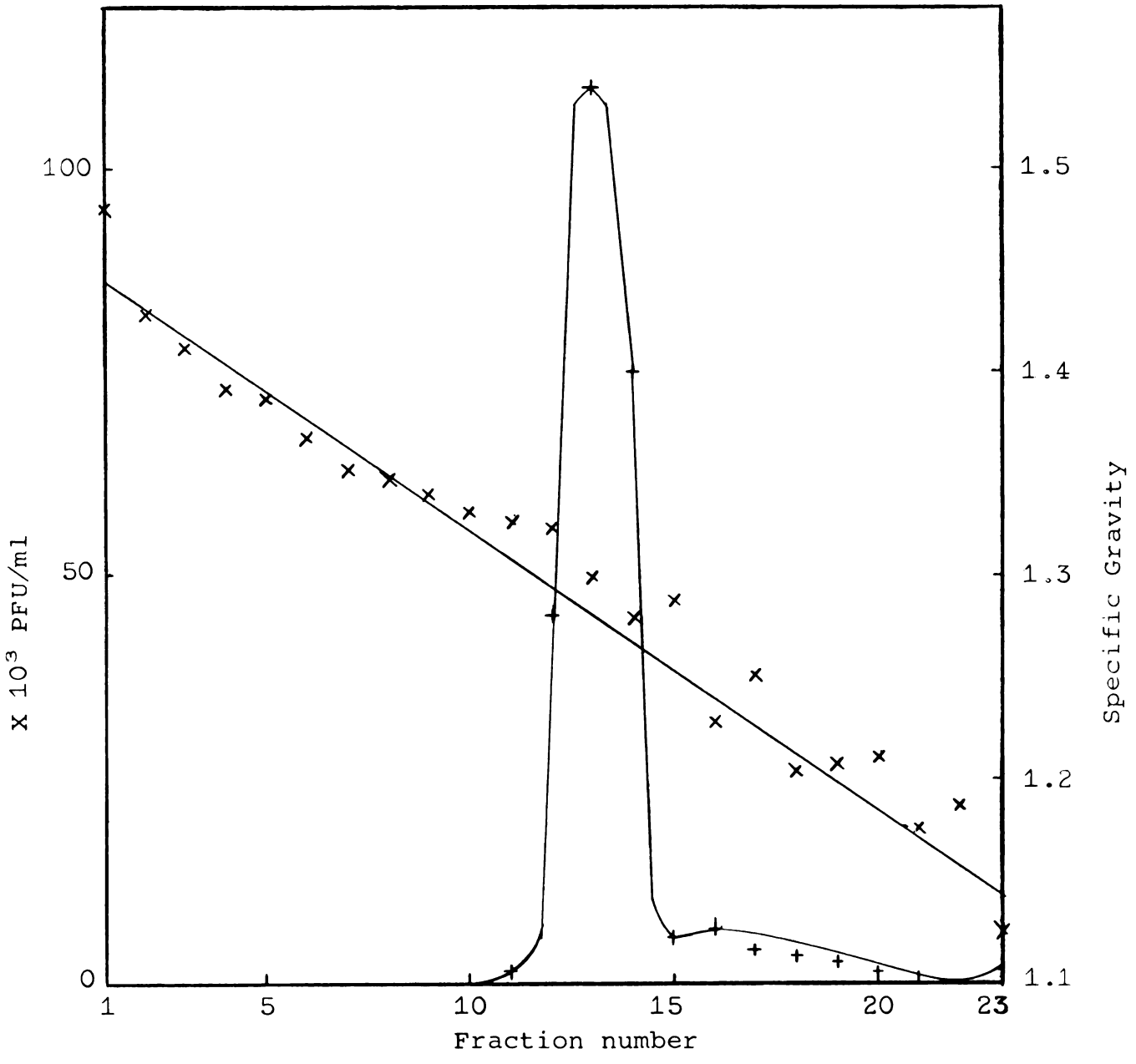

Figure 2. Cesium chloride density gradient centrifugation of IBV-42-113C.

+——+ Viral infectivity

x——x Specific gravity

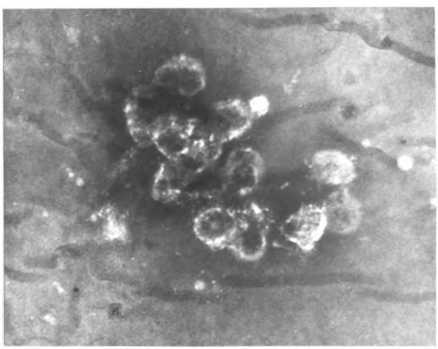

Figure 3: Electron micrograph of ether treated IBV-42-113C. Negatively stained with STA. X 120,000

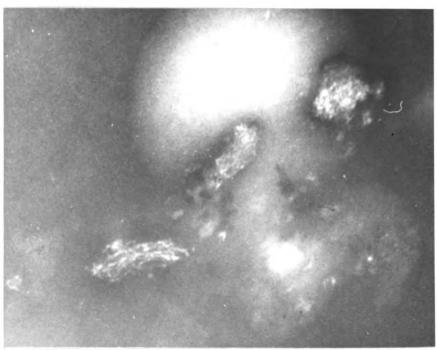

Figure 4: Electron micrograph of NP helix of IBV-42-113C released from the virus after ether treatment. Negatively stained with STA. X 120,000

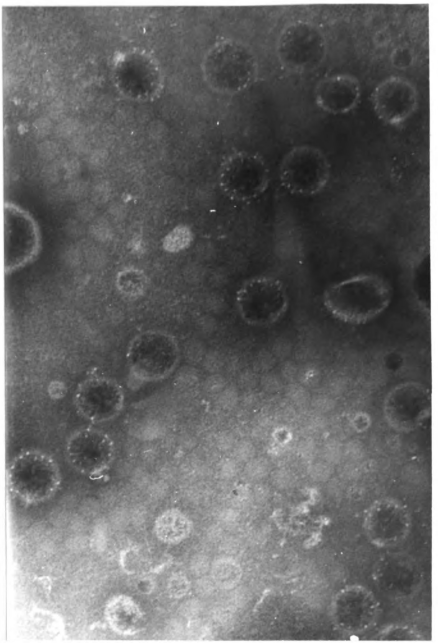

Figure 5: Electron micrograph of IBV-41-5 propagated in chicken embryo. Negatively stained with STA. X 120,000

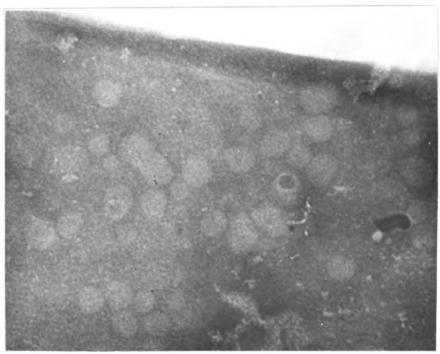

Figure 6: Electron micrograph of particles normally present in the allantoic fluid. Negatively stained with STA. X 120,000

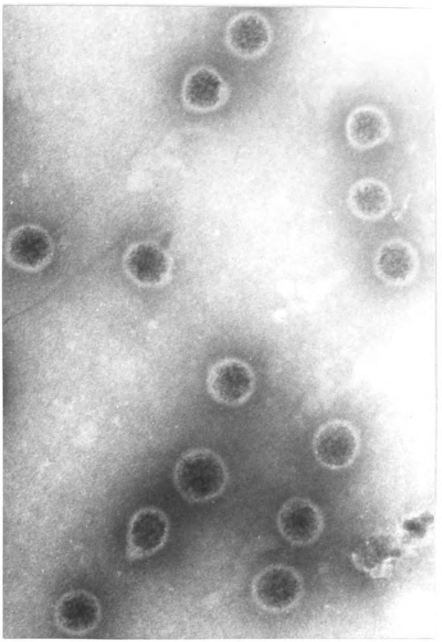

Figure 7: Electron micrograph of trypsin-modified IBV-41-5. Negatively stained with STA. X 120,000

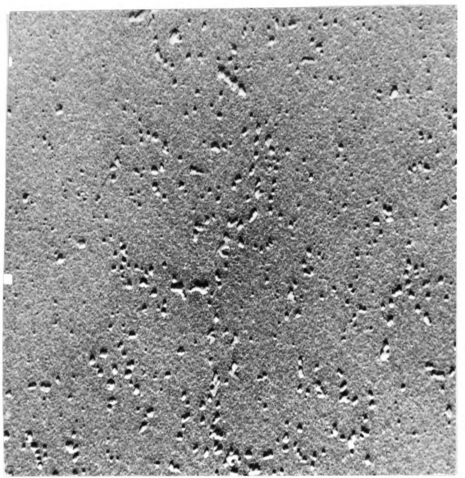

Figure 8: Electron micrograph of hemagglutinin fraction eluted from DEAE-cellulose column at 0.1 M NaCl. Shadow-cast at 30°. X 14,000

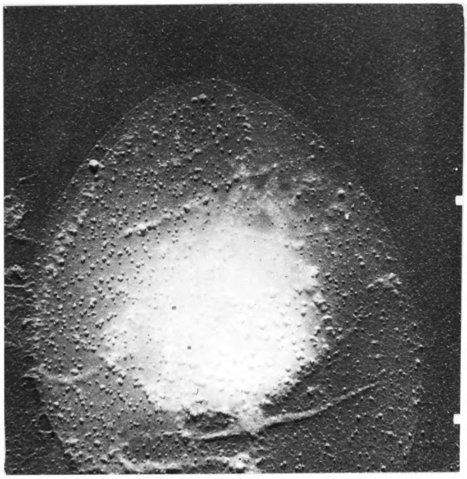

Figure 9: Electron micrograph of hemagglutinin particles adsorbed to the surface of chicken erythrocytes. Shadow-cast at 30°. X 14,000

Figure 10: Electron micrograph of chicken erythrocytes, 30 minutes after treatment with the hemagglutinin of the virus. Shadow-cast at 30^{o}. X 14,000

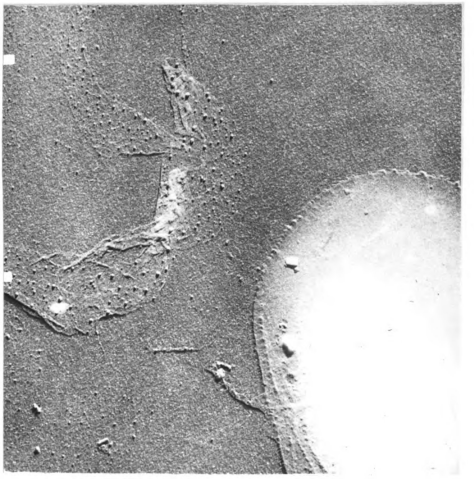

Figure 11: Electron micrograph of stroma of a chicken erythrocyte with hemagglutinin particles adsorbed to its surface. Shadow-cast at 30°. X 14,000

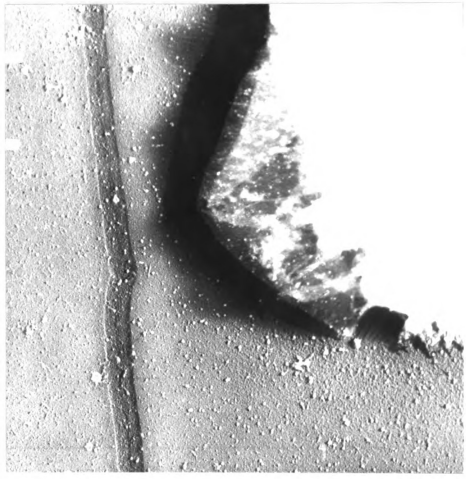

Figure 12: Electron micrograph of a portion of CEKC 30 minutes after treatment with IBV-42-113C. Shadow-cast at 30°. X 14,000

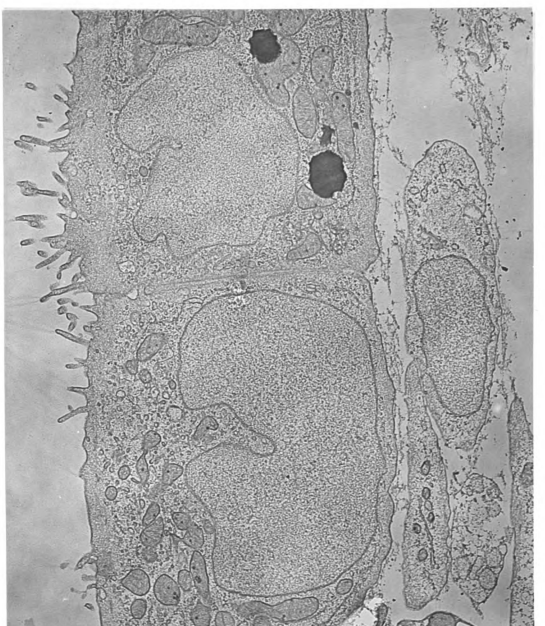

Figure 13: Electron micrograph of sections of non-infected CEKC. X 20,000

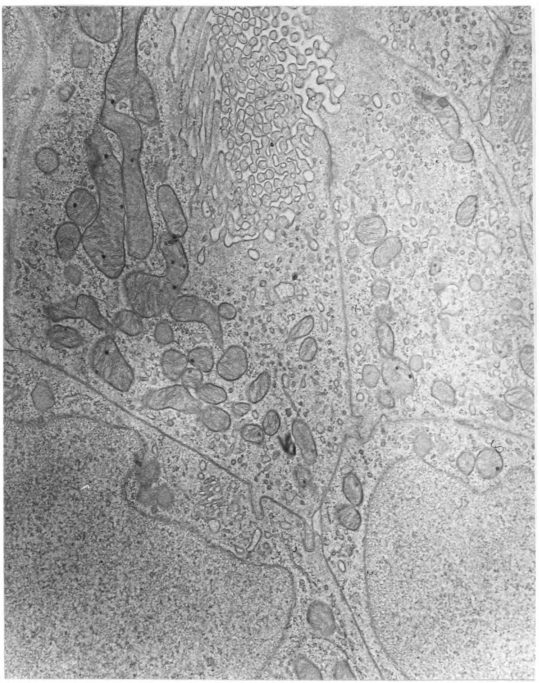

Figure 14: Electron micrograph of sections of CEKC culture 12 hours after infection with IBV-42-113C. X 20,000

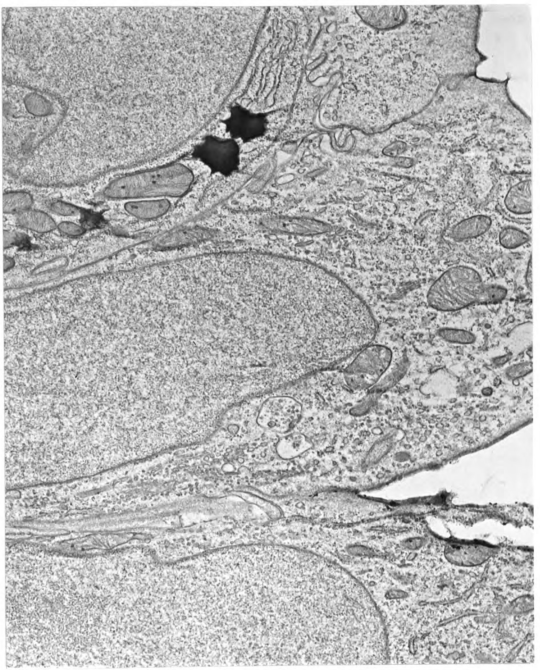

Figure 15: Electron micrograph of sections of CEKC culture 24 hours after infection with IBV-42-113C. X 20,000

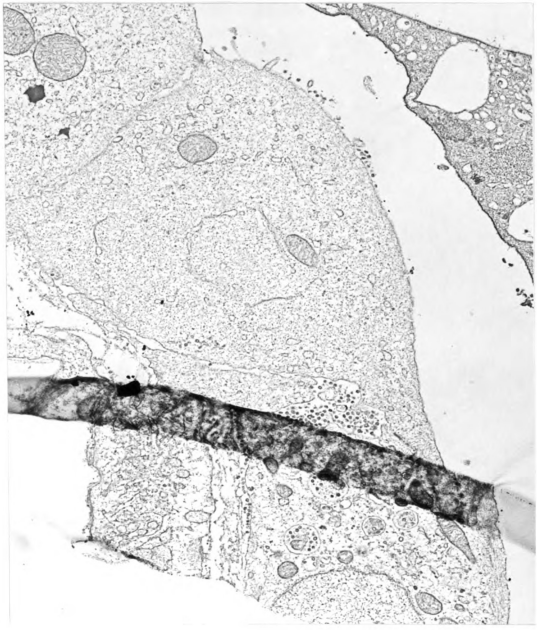

Figure 16: Electron micrograph of sections of CEKC culture 42 hours after infection with IBV-42-113C. X 20,000

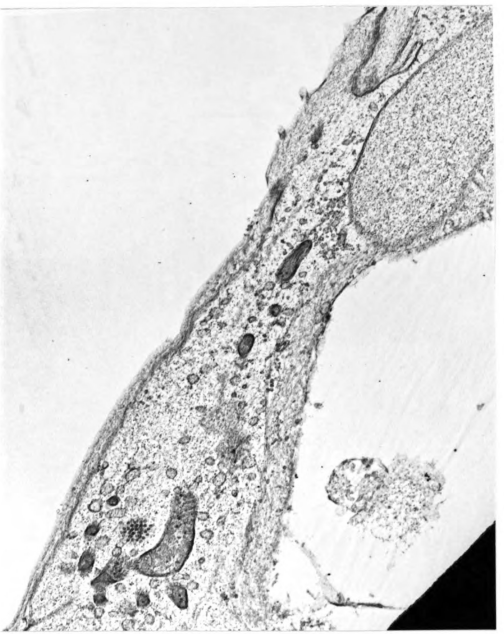

Figure 17: Electron micrograph of sections of CEKC culture 42 hours after infection with IBV-42-113C showing viral particles inside the cytoplasm, rupture of the nuclear membrane, and release of virus like structures into the cytoplasm. X 20,000

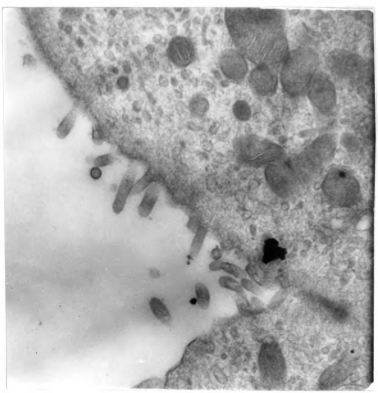

Figure 18: Electron micrograph of sections of CEKC culture 32 hours after infection with IBV-42-113C, indicating the release of viral particles by budding. X 24,000

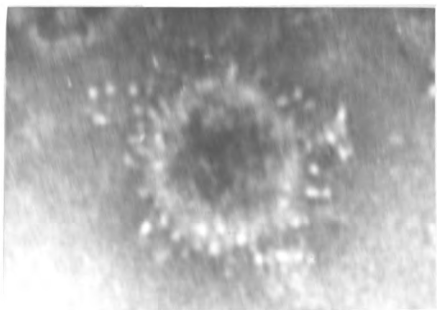

Figure 19: Electron micrograph of a single particle of IBV-42-113C. X 450,000

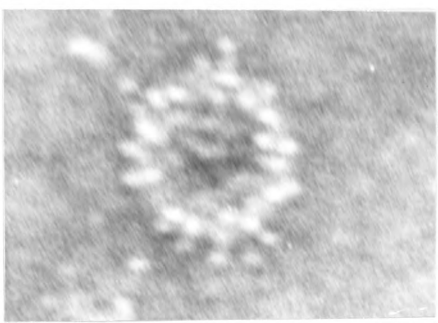

Figure 20: Electron micrograph of a single particle of IBV-41-5. X 450,000

DISCUSSION

Wilner (1964) classifies IBV with the myxoviruses which are generally RNA containing viruses, ether sensitive, have helical symmetry, size of 800 to 2,500 Å, many members agglutinate erythrocytes, and the virus matures at the cell surface. Berry et al. (1964) compared IBV to influenza as a model for the myxoviruses and found some features in common.

Infectious bronchitis virus contains RNA (Akers, 1963), and is ether sensitive (Mohanty, 1962; Akers, 1963). Treatment of the virus with ether releases three different antigens (Tevethia, 1964). Direct HA activity of the virus occurs only after treatment of the virus with trypsin (Corbo and Cunningham, 1959), ether, or selective elution from DEAE-cellulose columns (Biswal, 1965).

Ninety one per cent of infectious IBV-42-113C particles were sedimented at 74,000 x G for 60 minutes. Considering the size of the virus, 830-1,000 Å, sedimentation coefficient of 334 S, and specific gravity of 1.24 (Ellis, 1965) one would expect such a high recovery of viral particles. Ellis (1965), however, reported only a ten per cent recovery of infective particles.

Preparations of IBV-42-113C and IBV-41-5 partially purified by two cycles of centrifugation at 74,000 x G for 60 minutes each contained viral particles 830-1,000 Å in diameter with pear-shaped spikes on the surface and an internal NP helix. Further detailed study is needed to determine accurately the dimensions of the spikes and the NP helix.

The particulate hemagglutinin of the virus eluted from DEAE-cellulose columns was about the same size as the viral particle and adsorbed to the surface of erythrocytes within 30 minutes. Attempts to determine the surface structure and internal components of the hemagglutinin were not successful by negative staining but it is possible that it has the same structure as the infective viral particle.

Ether removed the spikes from partially purified IBV-42-113C grown in cell culture, disrupted the viral lipoprotein coat, and released the internal NP helix of the virus. Electron microscopy of ether treated virus after adsorption to erythrocytes was not performed and it is not known whether the disrupted viral particles or particles analogous to rosettes of influenza virus are responsible for hemagglutination by IBV. No rosette-shaped particles were observed in the ether treated viral preparations. It is possible that the NP helix released after ether treatment is one of the antigens of the virus, as reported by Tevethia (1964).

Trypsin removed the spikes from the surface of IBV-41-5 in allantoic fluid and digested the 330 Å particles with the virus in allantoic fluid and also in NAF alone. These particles might be the substances which normally inhibit direct HA activity of the virus. The inhibitory action of these particles is removed after treatment with trypsin. It is, therefore, permissible to assume that IBV-41 is capable of agglutinating erythrocytes but its activity is blocked by the allantoic inhibitor normally present with the virus. The inhibitor is digested by trypsin, has a surface charge density different from that of the virus, and is selectively eluted from DEAE-cellulose columns. The observation that treatment of viral preparations with ether also removed this inhibitor, along with its sensitivity to trypsin, suggests the glycolipoprotein nature of the inhibitor.

Allantoic fluid and other inhibitors of HA isolated from different biological sources have long been shown to prevent the HA activity of heat or periodate-inactivated virus commonly referred to as indicator virus. Heat or periodate selectively destroys neuraminidase but not the hemagglutinin of the virus. Non-treated virus is not, however, inactivated by these inhibitors. Neuraminic acid groupings present in the chemical composition of these inhibitors can compete with identical groupings on cell receptors for the virus. Neuraminidase present in influenza virus destroys the inhibitor, releases NANA and is still

able to attach to erythrocytes and remove their substrate. Indicator virus and viruses lacking neuraminidase are not able to destroy the inhibitor and their HA activity is blocked.

The observation that allantoic fluid inhibitor is active against the hemagglutinin of IBV may indicate indirectly a lack of neuraminidase by the virus as has been previously suggested by Berry et al. (1964). Biswal (1965), however, reports some neuraminidase-like activity to be associated with the virus. It is possible that the enzyme is present in a small amount or it is not stereospecific for the inhibitor molecule.

Specific immune and normal chicken sera inhibit the HA activity of trypsin modified IBV-41 (Muldoon, 1960) to the same extent. This again might indicate the absence of neuraminidase in the virus allowing an inhibitor present in normal serum to block the HA activity of the virus. Hemadsorption of CEKC infected with IBV-41 was reported by Biswal (1965) and is related to the HA activity of the virus. Probably there is no HA inhibitor in the cell culture system for IBV and the virus is able to reveal its HA activity by causing the hemadsorption of the cells.

Ether sensitivity of IBV and several other features in common with the myxoviruses lead to the assumption that the lipoprotein coat of the virus is of cellular origin and that the virus matures on the surface of the cell. Akers

(1963) likewise, proposed the possibility of formation of complete particles of IBV at the cell surface based on quantitative infective studies of the development of the virus and by ultra-violet spectroscopy.

In the present study viral particles were observed in the cytoplasmic matrix of infected cells 12 hours after infection and accumulated in cytoplasmic vesicles within 24 hours after infection. The vesicles containing IBV are probably similar to the structures stained 48 hours after infection with specific fluorescent antibody as reported by Mohanty (1964). Cytoplasmic vesicles containing the virus have been observed with cells infected with adenovirus (Dales, 1962), western equine encephalitis (Morgan et al., 1961), mouse leukemia (Parsons, 1964) and are reported by Mandel (1962) to be due to envagination by cell membrane related to pinocytosis. The presence of complete particles of IBV in the cytoplasmic matrix and vesicles separates this virus from influenza virus which is only detected on the cell membrane. These two viruses, however, have several other features in common. There is some morphological resemblance between them as to the size, presence of spikes, and an internal NP helix. Both are RNA viruses, ether sensitive, agglutinate erythrocytes and have been reported to contain neuraminidase.

SUMMARY

1. Infectious bronchitis virus in allantoic fluid and in cell culture media is a sphere of 830 to 1,000 Å in diameter. The virus has a lipoprotein coat with pear-shaped spikes on its surface and an NP helix internal component.

2. The virus has a specific gravity of 1.29 as determined by cesium chloride density gradient centrifugation.

3. Hemagglutinin of IBV as eluted from DEAE-cellulose columns is particulate in nature, has the same dimensions of the viral particle, and adsorbs to chicken erythrocytes within 30 minutes.

4. A glycolipoprotein inhibitor of HA, 330 Å in diameter, is detected in NAF and AF containing the virus. This inhibitor has a surface charge density different from that of the virus and can selectively be separated from the hemagglutinin DEAE-cellulose column chromatography.

5. Trypsin removes the spikes from the viral coat and destroys the 330 Å inhibitor of HA from AF containing the virus.

6. Ether removes the spikes, disrupts the lipoprotein coat, and releases the NP helix of the virus.

7. Viral particles, 900 Å in diameter, are observed in the cytoplasmic matrix and cytoplasmic vesicles of cells 12 hours after infection. There is some indication of intranuclear replication of the virus.

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