STUDIES OF THE ANTIGENS OF INFECTIOUS BRONCHITIS VIRUS

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THESIS

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ABSTRACT

STUDIES OF THE ANTIGENS OF INFECTIOUS BRONCHITIS VIRUS

by Satvir Singh Tevethia

The antigenic nature of infectious bronchitis virus (IBV) was investigated by the immunodiffusion technique using anti-IBV chicken serum. The virus in allantoic fluid contained at least 3 antigens, designated 1, 2 and 3, which were identical to the antigens from IBV-infected chorioallantoic membrane (CAM). Sensitivity of the virus to ether and sodium deoxycholate indicated the presence of lipids in the virus. When virus purified 800 fold on the basis of specific infectivity by centrifugation was treated with ether and heated at 100 C for 30 minutes, it disintegrated into antigenic units which were identical to the antigens 1, 2 and 3.

Antigens 1, 2 and 3 were not sedimented at 109,000 x g after one hour. Infective virus was sedimented under the same conditions. There was a parallel release of both infectious virus and the antigens into the extracellular fluid of the CAM cultivated in vitro. Antigens 1 and 3 were thermolabile. Antigen 2 was thermostable. Antigen 2 is a ribonucleoprotein as indicated by its sensitivity to

ribonuclease, trypsin and pepsin. Antigen 1 was susceptible to trypsin and pepsin. DNase had no effect on either of the antigens. Enzyme sensitivity of antigen 3 could not be determined due to its low concentration. Antigen 1 was not retained by a 10 mu filter. Antigen 2 was retained by a 10 mu filter but not by a 50 mu filter. Antigen 3 and infective virus were retained by a 100 mu filter.

Antigenic differences between the Massachusetts 41, Connecticut 46 and Beaudette embryo adapted 42 cultures of virus were not detected by the immunodiffusion test. Cross neutralization tests did detect antigenic differences.

Reaction of noninfective antigens with anti-IBV 41 chicken serum did not reduce the capacity of the serum to neutralize viral infectivity. This indicates that the antigens do not compete with the virus for neutralizing antibodies.

Antigens 1 and 2 were fractionated on DEAE-cellulose. Antigens 1 and 2 were eluted by 0.05 and 0.15 M NaCl, respectively, in 0.02 M phosphate buffer, pH 7.2. Approximately 96% of the infective virus was eluted by 0.45 M NaCl whereas 4% of the virus was eluted by 0.90 M NaCl.

Using cesium chloride density gradient centrifugation, the virus had a density of 1.23. Antigens 1 and 2 had densities of 1.13 and 1.17, respectively.

It is proposed that IBV might be considered to be a member of the Myxovirus group based on the following criteria associated with this group: (1) Sensitivity to ether and sodium deoxycholate, (2) ribonucleoprotein complex containing RNA, (3) forms syncytia in cell culture, (4) has a lipoprotein coat and (5) possesses surface projections similar to influenza viruses.

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Satvir Singh Tevethia

A THESIS

Submitted to
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DEDICATED

TO

MY FAMILY

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INTRODUCTION

Although precipitation of IBV by specific immune serum has been demonstrated (Woernle, 1959, 1960 and 1962; Witter, 1962; Tevethia, 1962), the precipitation test is not used extensively for diagnostic purposes. At least 2 antigens have been demonstrated with infectious bronchitis virus using the precipitation test but the specificity of these antigens and their possible relationship to the virus has not been adequately investigated.

The objectives of the present study were: (1) to study the relationship of precipitating antigens to the virus, (2) to characterize these antigens physically, chemically and immunologically and (3) to partially characterize the virus itself.

LITERATURE REVIEW

Infectious Bronchitis Virus

Infectious bronchitis virus, the causative agent of a highly contagious respiratory disease in chickens, is a sphere with a diameter of 60 to 80 mu (Nazerian, 1960). The virus multiplies readily in 10 day old embryonating chicken eggs by various routes of inoculation. The virulence of the virus for chickens is modified during serial passage of the virus in chicken embryos. Low egg passage virus produces gross pathological changes in chicken embryos whereas high egg passage virus causes embryo mortality (Cunningham, 1957). The Beaudette embryo-adapted culture (42) can be cultivated in the isolated chorioallantoic membrane (Ferguson, 1958; Ozawa, 1959), and in chicken embryo liver (Fahey and Crawley, 1956), and kidney cell (CEKC) cultures (Spring, 1960). The virus produces plaques on CEKC cultures (Wright and Sagik, 1958; Cunningham, 1963b).

The virus has an eclipse phase of 4 hours in CEKC. After the 6th hour there is an exponential increase of the extracellular virus up to 36 hours followed by a phase of decline to 72 hours. Cell associated virus follows a

similar pattern but the maximum amount of virus is present within 24 hours. Virus multiplication is accompanied by the formation of syncytia which parallel the increase in released and cell associated virus. Accumulation of virus-specific ribonucleic acid (RNA) in the syncytial cytoplasm can be demonstrated within 24 hours using acridine orange stain. The formation of syncytia is supressed by specific immune serum. The formation of syncytia and viral synthesis are inhibited by D L-p-fluorophenylalanine added to the cultural medium 12 hours prior to the inoculation of cells with IBV. Aminopterin (4-aminopteroyl-L-glutamic acid), has no effect on the development of the virus (Akers, 1963).

Immunofluorescence studies (Mohanty et al., 1964) with the Beaudette culture of the virus showed that viral antigen first appears in the nucleus 7 hours post inoculation. From 36 to 48 hours, the antigen can be detected in the cytoplasm. According to Stultz (1962) the viral antigen can first be detected in the cytoplasm as early as $2\frac{1}{2}$ hours after infection.

Infectious bronchitis exists in two phases: the thermostabile original (O) phase as originally isolated from chickens and the thermosensitive derivative (D) phase resulting from serial passage of the virus in chicken embryos. The O phase can be separated from a O-D phase virus by differential inactivation of the D phase at 56 C. Only the O phase is antigenic and pathogenic to chickens (Singh, 1960).

Two antigenic types of IBV, Massachusetts (41) and Connecticut (46), are generally recognized and can be differentiated by reciprocal neutralization tests (Jungherr et al., 1956; Hofstad, 1958). The degree of cross neutralization between Connecticut and Massachusetts types is related to the embryo passage of the type of virus. According to Oshel (1961) cross neutralization between these types does not occur with the low embryo passage virus but does occur with higher embryo passage virus.

Two virus-specific antigens have been demonstrated in IBV 42, 41 and 46 in allantoic fluid by means of the agar gel diffusion using anti-IBV rabbit serum. The antigens are heat stabile but are susceptible to trypsin. Massachusetts and Connecticut types possess one common and one unrelated antigen which can be differentiated by reciprocal agar gel diffusion tests (Tevethia, 1962).

Infectious bronchitis virus agglutinates chicken erythrocytes only after treatment of the virus with trypsin. The hemagglutinin is heat stabile (Corbo and Cunningham, 1959; Muldoon, 1960). Trypsin modified IBV reduces the electrophoretic mobility of chicken erythrocytes by 21% as compared to 41.9% reduction by influenza virus (Biswal, 1963).

The virus is ether sensitive (Akers, 1963; Mohanty, 1964).

Soluble Antigens

Soluble antigens are serologically specific for certain components of the virus but they are noninfectious. It has been suggested that the soluble antigens are viral structures produced in large excess during virus multiplication that are not incorporated into the complete viral particles (Wilcox and Ginsberg, 1963a). The soluble antigens can be isolated either from the virus-infected cells (Wilcox and Ginsberg, 1961) or from degradation of the virus (Wilcox and Ginsberg, 1963b; Hoyle, 1952; Lief and Henle, 1956). The soluble antigens are sedimented more slowly than the infective virus in the ultracentrifuge. Soluble antigens may be used for complement fixation, precipitation and hemagglutination tests (Lennette, 1959).

Craigie (1932) was the first to demonstrate soluble antigens from vaccinia virus using precipitation and complement fixation tests. The antigens could be separated from the viral particles by filtration and centrifugation.

A nucleoprotein antigen extractable from vaccinia virus was demonstrated by Smadel *et al.*, (1942).

At least 3 soluble antigens are released from adenovirus-infected cells (Wilcox and Ginsberg, 1961). The 3 antigens, 1, 2 and 3 based on the order of elution from diethylaminoethyl (DEAE) cellulose, have a diameter of 10,

17 and 8 mu, respectively. Antigens 1 and 3 are nucleoprotein (Allison et al., 1960). Antigen 1 is type specific while antigen 3 is group specific (Wilcox and Ginsberg, 1961). Antigen 2 causes cytopathogenic effects in tissue culture. Wilcox and Ginsberg (1963b) investigated the antigenic relationship of virus structural proteins to the virus specific soluble antigens from infected cells. The purified virus after dialysis against 0.1 M carbonate-bicarbonate buffer at pH 10.5 for 4 days disintegrates into subunits with the loss of infectivity and a change in the buoyant density from 1.3349 to 1.2832. The antigens released from the purified virus are immunologically identical to the soluble antigens from infected cells. On the basis of cesium chloride density gradient centrifugation, soluble antigens from purified virus after dialysis at pH 10.5 have a mean buoyant density, 1.2832, identical with the purified soluble antigens from infected cells. Antigenic analysis of the soluble antigens indicates that these are surface antigens. The subunits of the purified virus particles are morphologically identical with the soluble antigens from virus-infected cells (Wilcox et al., 1963).

Soluble antigens have been described for all Myxoviruses. Two antigens are associated with all types of influenza viruses: a type specific soluble antigen and a strain specific V antigen. The soluble antigen is present

in large amount in the virus-infected tissue. Hoyle (1952) demonstrated that purified influenza virus after treatment with ether disintegrates with the release of S antigen and the hemagglutinin. Studies of Lief and Henle (1956) support these findings. The soluble antigen is a ribonucleoprotein and is sensitive to ribonuclease and trypsin. Soluble antigen from influenza virus is serologically identical with the soluble antigen from virus-infected cells. The nature of these antigenic components is reviewed by Lennette (1959); Schmidt and Lennette (1961); Schafer (1963) and Hummeler (1963).

Two type-specific antigenic components (D or N and C or H) have been reported in poliovirus-infected tissue cultures (Hummeler and Hamparian, 1958; Mayer et al., 1957). The complement fixing activity was sedimented at high speed centrifugation (Schmidt and Lennette, 1956). Black and Melnick (1955) reported a nonsedimentable soluble complement fixing antigen present in formalized poliovirus.

According to Scharff et al. (1964) a soluble antigen with a sedimentation constant of 2S is released upon treatment of poliovirus with guanidine. The antibody against the guanidine-degraded virus can be used to detect a soluble protein with a molecular weight of 80,000-100,000 in the infected cells which is a precursor of the virus particles. The antigen is not sedimented at 100,000 x g after two hours.

According to Rott et al. (1963), serologic and electron microscopic evidence indicates that soluble antigen extracted from NDV and Sendai virus-infected cells is identical to the soluble antigen of the virus itself. The soluble antigen in the infected tissue represents the excess which is not incorporated into the virus particles.

Group and type-specific Coxsackie virus antigens have been investigated by means of the agar gel diffusion test. The antigens can be separated by cesium chloride density gradient centrifugation (Schmidt et al., 1963).

A soluble precipitating antigen from hog cholera virus propagated in tissue culture has been demonstrated by Pirtle (1964). The antigen is heat sensitive and is separable from the virus by DEAE-cellulose chromatography.

Stone (1960) reported 2 specific precipitating antigens in the lymph nodes of steers infected with rinderpest virus. One of the antigens is heat sensitive. According to White and Cowan (1962) the antigen is soluble in nature since it could not be sedimented by centrifugation after 2 hours at 100,000 x g.

Immunodiffusion

Oudin (1946) introduced the technique of single diffusion in one dimension and suggested the possibility of double diffusion in one dimension. The double diffusion technique in two dimension was developed by Ouchterlony (1948) and Elek (1948).

The principles and methods of immunodiffusion have been reviewed by Oudin (1946), Ouchterlony (1958; 1961; 1962), Crowle (1960; 1962) and Kabat and Mayer (1961). The double diffusion test is useful in resolving multiple precipitating systems into their individual components and comparing 2 antigens against the same antiserum or two antibodies against the same antigen.

Bjorklund (1952) developed the inhibition plate technique based on the principle that on pretreatment of the diffusion medium with the sufficient amount of a component of a complex immunologic system, the subsequent appearance of the precipitates corresponding to the particular component could be completely inhibited.

The single and double diffusion techniques have been used extensively for antigenic analyses of influenza virus (Jensen and Francis, 1953), pox virus antigens (Gispen, 1955), fowl pox virus (Wittmann, 1958), swine fever virus (Forsek, 1958), foot and mouth disease virus (Bodon, 1955; Brown and Crick, 1957 and 1958), poliovirus (Le Bouvier, 1957; Le

Bouvier et al., 1957; Grasset et al., 1958; Scharff et al., 1964), rinderpest virus (White, 1958; Stone, 1960; White and Cowan, 1962), infectious bronchitis virus (Woernle, 1959, 1960, 1961; Witter, 1962; Tevethia, 1962), Coxsackie virus (Schmidt and Lennette, 1962a and 1962b) and ECHO viruses (Middleton et al., 1964).

Mata and Weller (1962) described an agar cell culture precipitation test to detect the release of viral antigens from the infected cells cultivated in the agar. This test has been used to study precipitating and complement fixing antigens of vaccinia virus, adenovirus, herpes virus and enteroviruses.

Ragetli and Weintraub (1964) studied the interaction of plant viruses with homologous antibody in agar gel under the influence of an electric field. Precipitin lines were obtained in 50 to 90 minutes. This technique is named as "Immuno-osmophoresis" since the movement of the antibody is due to endosmotic flow. Immuno-osmophoresis is claimed to be more sensitive and faster than Ouchterlony's immunodiffusion method.

MATERIALS AND METHODS

Viruses

Cultures of IBV identified by the Michigan State University repository code numbers as 41, 42 and 46 were used.

Culture 41 was isolated by Van Roekel in 1941 and had been maintained by serial passages in chickens. The virus was in the 4th chicken embryo passage and had a titer of 10^7 to 10^8 embryo infectious dose$_{50}$ (EID_{50}) at the time of this study.

Culture 42 had been through hundreds of passages in chicken embryos. The EID_{50} was 10^7 to 10^8. When tested in chicken embryo kidney cell cultures, the titer was from 10^5 to 10^6 plaque forming units (PFU) per ml.

Culture 42 adapted to CEKC cultures by Spring (1960) was in the 109th passage. The virus is identified as IBV 42-110 C. The titer was 10^6 to 10^7 PFU/ml.

Culture 46 was isolated by Jungherr *et al*. (1956) from a commercial vaccine and was in the 12th egg passage. The titer was 10^6 to 10^7 EID_{50}.

Cell Culture

The procedure for cell culture was similar to that described by Cunningham (1963). The kidneys from 17 to 18 day old chicken embryos were removed aseptically and were washed several times with Hanks' balanced salt solution (BSS) containing phenol red. The kidneys were then cut into 1 to 2 mm pieces and were washed free of blood clots and other tissue debris. The washed pieces of kidneys were then transferred to a 500 ml trypsinizing flask containing a Teflon covered magnet. Ten ml of 0.25% trypsin in BSS, pH 7.8 to 8.2, per pair of kidney was then added to the flask and the contents were agitated by means of a magnetic stirrer for one hour at room temperature. The cell suspension was filtered through two layers of cheese cloth and then centrifuged at 200 x g for 6 to 8 minutes. The trypsin was decanted and the cells were suspended in fresh BSS. The process was repeated. After the BSS was decanted, one ml of packed cells was suspended in 100 ml of growth medium consisting of medium 199 supplemented with the vitamins, and amino acids of Eagle's basal medium and with L-glutamine, 0.1% sodium bicarbonate, and 5% new born calf serum (Grand Island Biological Co., Inc., Grand Island, N.Y.) and 100 units of penicillin, 100 ug of streptomycin and 50 units of mycostatin per ml. The final concentration was approximately 1×10^7 cells per ml. Plastic, 15 mm x 60 mm, tissue

culture petri dishes (Falcon Plastics) were seeded with 4 ml of the cell suspension. All incubation was at 37 C in an atmosphere of 8% CO_2 and 80 to 85% humidity. A satisfactory confluent monolayer of cells was formed after 48 to 60 hours.

Propagation of Viruses

Cultures 41, 42 and 46 were propagated by inoculating 10 day old embryonating chicken eggs with 0.2 ml of a 10^{-2} dilution of the virus contained in allantoic fluid. Allantoic fluid was collected from living embryos 24 hours post inoculation with IBV 42 but 36 hours with IBV 41 and 46. The embryos were chilled for at least 4 hours before collecting the fluids which were pooled and stored at -60 C until used. Chicken embryo kidney cultures (5 x 10^6 cells/ml) were used to propagate IBV 42-110 C. The cell monolayer was washed once with 3 ml of BSS without phenol red and inoculated with 0.5 ml of a 10^{-1} dilution of the stock virus. After 90 minutes at 37 C, 4 ml of growth medium was added to each plate. The plates were incubated for 36 to 40 hours at which time the cytopathic effects were most pronounced. The extracellular fluids were pooled and stored at -60 C.

Virus Assay

Plaque Assay

Cold BSS without phenol red was used as the diluent. The cells were washed once with 3 ml of BSS and were then inoculated with 0.5 ml of the appropriate serial dilution of the suspension of the virus. Four dishes were used per dilution unless indicated otherwise. The virus was allowed to adsorb to the cells for 90 minutes. After incubation, the inoculum was poured off and the cells were overlayed with 4 ml of 0.9% Difco Noble agar in cultural medium. After incubation for 72 hours, 0.5 ml of a 0.1% solution of neutral red in PBS was added to each plate. The plates were incubated at 37 C for one hour and then at 4 C for at least 2 hours before plaque counts were made. The titer of the virus was expressed as PFU/ml.

Virus Assay in Chicken Embryos

The EID_{50} of IBV 42, 41 and 46 was determined by inoculating 10 day old embryonating chicken eggs with serial 10 fold dilutions of the viruses. Five embryos were inoculated per dilution. The positive response for IBV 42 was embryo mortality. For IBV 41 and 46, the positive responses were embryo mortality, curling of the embryos, deformed feet compressed over head, thickened amniotic membrane and mortality. The titers of the viruses were calculated according to the formula of Reed and Muench (1938).

Antigens

Antigens from Viral Allantoic Fluid

Preliminary studies conducted in the present study on precipitation of IBV antigens by homologous chicken antiserum in agar gel indicated that the virus in allantoic fluid is not a good source of antigens. Concentration of the virus with polyvinylpyrolidone (PVP) provided a good antigen (Tevethia, 1962). In the present study, the virus was concentrated 10 fold by dialyzing against polyethylene glycol (Carbowax 4000, Union Carbide Chemical Company).

Antigens from Viral-Infected CAM

Chicken embryos were inoculated with 0.2 ml of the 10^{-2} dilution of IBV 42 stock virus via the allantoic cavity. After 20 hours the CAMS were collected, washed 3 times with physiological saline solution and suspended in 2 ml of 0.02 M phosphate buffered saline solution (PBS), pH 7.2, per membrane. The membranes were then homogenized in a chilled Waring-blendor for 5 minutes. Forty ml of the homogenate was used for sonification (Bronwill Scientific Division/Will Corporation, Rochester, New York) at 20 kilocycles per minute at 23 to 25 C for intervals from 20 to 60 minutes. The sample was centrifuged at 600 x g for 30 minutes. The supernatant fluid was removed and the sediment was resuspended in half the original volume in PBS. Both samples were tested for precipitating antigens. The immunodiffusion studies

showed that 60 minutes of sonication produced the maximum precipitation as judged from the intensity of the precipitin band in agar gel. The sediment also produced lines of precipitation indicating that viral antigens are firmly bound to the cellular material. The supernatant fluids of the sonified CAM were lypholized in 5 ml quantity, sealed and stored at -4 C until used. The lyophilized samples were reconstituted with one ml of distilled water before use.

Embryos were inoculated with one ml of undiluted virus via the CAM route using the artificial air sac method. The CAMs were harvested after 20 hours and treated as described above. On the basis of the intensity of line of precipitation more antigen was produced by this method of propagation of the virus than by the two previously described methods. These results are similar to those reported by Issacs and Fulton (1953) that infection on the chorionic side of the membrane by influenza virus results in the preferential production of a large amount of soluble complement fixing antigens.

Antigens from Partially Purified Virus

Twenty ml of peroxide free anaesthetic ether and 40 ml of virus concentrated by differential centrifugation (see Centrifugation) were placed in an Erlenmeyer flask and mixed with a magnetic stirrer at room temperature for 2 hours.

The mixture was centrifuged at 600 x g for 10 minutes to separate the aqueous and ether phases. The ether phase and the cloudy interphase were removed with a pipette and dry nitrogen was bubbled through the aqueous phase to remove the remaining ether. One ml of PBS was added to the ether phase and the ether was removed by dry nitrogen. Viral antigens could not be detected in the ether phase. The interphase and the aqueous phase contained antigens. The latter two phases were pooled.

Controls

The controls included normal allantoic fluid (NAF), normal CAM and ether treated normal allantoic fluid.

Antiserums

Antisera against IBV 41, 42 and 46 were produced in 4 to 6 month old Single Comb White Leghorn cockerels by the method of Hofstad (1958). The chickens were divided into 3 groups of 10 each. Groups I, II and III were inoculated intratracheally with 0.2 ml of undiluted IBV 41, 42 and 46, respectively. Four weeks later, 5 ml of the viruses were injected intramuscularly and birds were bled 10 days later. The chickens were fasted for 24 hours prior to bleeding. The sera from individual birds were pooled for each virus type, centrifuged and stored at -20 C. The sera collected from 2 birds from each group prior to virus exposure were

negative for IBV precipitating antibodies. Anti-IBV 41 and 46 sera prepared in the above manner failed to precipitate homologous antigens. Only the serum against IBV 42 gave a positive precipitin test. All three antisera possessed neutralizing antibodies against the homologous and heterologous virus types (Table 7).

Since precipitating antibodies were not detected in anti-IBV 41 and 46 sera, a preliminary experiment was set up in which 20 birds were inoculated intratracheally with 0.2 ml of IBV 41. Starting on the 7th day, blood was collected every other day through the 29th day. Precipitating antibodies were detected from 7 to 23 days after infection but not 25 to 29 days after infection. Based on the intensity of precipitation, antibodies were at the highest concentration 12 to 18 days after infection.

Antiserum containing a high concentration of precipitating antibody against IBV 41 was prepared by inoculating 15 birds with 0.2 ml of undiluted virus intratracheally. The birds were bled 15 days postinoculation. The sera giving a positive precipitin test were pooled and stored at -20 C. Attempts to prepare a potent precipitating serum against IBV 46 were unsuccessful.

Immunodiffusion Techniques

Preparation of Immunodiffusion Plates

Fifty ml of 0.15 M phosphate buffer, pH 7.2, containing 8% sodium chloride and 0.75% IONAGAR no. 2 (Consolidated Laboratories Inc., Chicago Heights) in 50 ml of glass double distilled water were autoclaved separately at 121 C for 15 minutes. After cooling to 60 C, the two solutions were mixed. Ten ml of the diffusion medium was uniformally spread on a precleaned $3\frac{1}{4}$ inch x 4 inch Kodak lantern cover glass. Only 2.5 ml of the diffusion medium was poured on a clean Leitz 2 inch x 2 inch cover glass plate. The plates were stored in a humid chamber at 4 C for at least 4 hours before used.

The templates used for making the immunodiffusion well patterns depended on the type of test employed. The size and the distance between the wells also depended upon the conditions best suited for a particular test. The time of incubation also varied with the system used but was usually from 6 to 10 days. At the end of the test, the plates were washed for 36 hours with 3 changes of PBS to remove the unreacted components. After washing, the plates were dried by putting a Whatman No. 1 filter paper on the agar surface and allowing them to stand at room temperature for 4 to 5 hours. After removing the filter paper from the agar surface, the slides were then stained in triple stain

containing thiazine red, light green and amido black (Crowle, 1962) for 15 minutes. The slides were then washed in 3% acetic acid to remove the nonspecific stain. The destaining solution was changed several times until the background was clear. The slides were dried at 37 C.

Since some of the precipitation lines could not be suitably photographed, schematic drawings of the precipitations patterns were drawn to demonstrate the various types of precipitation reactions.

Optimum Conditions for Immunodiffusion Tests

Preliminary studies were carried out to establish the optimum conditions for the precipitation of viral antigens by the antibody in agar gel. Four variables were investigated: (1) incorporation of 1/10,000 merthiolate in the diffusion medium; (2) sodium chloride concentration, 0.8% and 8%; (3) incubation at 4 C and room temperature; and (4) incorporation of 5% normal chicken serum in the medium. Constant amounts of antigen from IBV-infected CAM and anti-IBV 41 chicken serum were used. The wells of the diffusion plates were filled 3 times at 6 hour intervals. The plates were incubated in a humid chamber. Incorporation of 1 10,000 merthiolate in the diffusion medium inhibited the viral antigen antibody precipitation. Precipitation seemed to be more complete at 4 C than at room temperature.

Normal chicken serum in the medium greatly enhanced the intensity of precipitation. Sodium chloride, 8%, was essential for optimum precipitation. These studies established the optimum conditions for the immunodiffusion tests: 0.75% agar, 8% sodium chloride in 0.15 M phosphate buffer at pH 7.2, and 4 C for incubation. The use of 5% normal chicken serum was not continued since it was difficult to remove even after repeated washing of the plates in PBS. When specific lines of precipitation were stained with basic dyes, the serum remaining in the agar provided a nonspecific background stain thus interfering in the interpretation of specific lines. The plates were easily contaminated.

Ether Sensitivity

Forty ml each of IBV 42 and IBV 42-110 C was clarified by low speed centrifugation and placed in a sterile Erlenmeyer flask containing a Teflon covered magnet. A 5 ml sample of each was removed and then 20 ml of ether was added to the virus. The sample was mixed with a magnetic stirrer at 4 C at room temperature. Samples of 5 ml each were withdrawn at 5, 10, 15, 30, 60 and 120 minutes and were centrifuged immediately at 600 x g for 15 minutes to separate the ether and the aqueous phases. The ether phase was removed with a pipette. The remaining ether in the aqueous phase was removed by passing dry nitrogen through it continuously

until the aqueous phase was free of ether. The samples were then tested for infectivity using the plaque method. A control of virus alone was also set up at 4 C and room temperature and the virus was agitated with magnetic stirrer. One initial and the other final sample at the end of the experiment was withdrawn from the control flasks.

Sensitivity of the Virus to Sodium Deoxycholate

A 5% solution of sodium deoxycholate (Consolidated Laboratories, Inc., Chicago Heights) in PBS was diluted to contain 1%, 0.8%, 0.6%, 0.4%, 0.2% and 0.1% respectively in separate tubes. Two ml of the above concentrations of sodium deoxycholate were mixed with 2 ml of IBV 42 in allantoic fluid clarified at 600 x g. The virus control consisted of an equal amount of virus and PBS. After incubation for 10 minutes at room temperature, the sodium deoxycholate-virus mixtures were diluted in cold BSS and titrated for residual virus infectivity by the plaque method.

Filtration

Sterile Millipore filters of 450, 300, 100, 50 and 10 mu average pore diameter were used. Three ml of PBS was passed through the filters to satisfy the adsorption capacity of the filter followed by 3 ml of the allantoic fluid virus clarified by centrifugation. The virus samples were

titrated for the residual infectivity by the plaque method.

Antigens from the infected CAM and ether treated virus were also passed through Millipore filters of the same pore diameter and detected by Agar gel diffusion.

Protein Determination

Protein was determined by spectrophotometry according to the method of Lowry et al. (1951).

Centrifugation

One hundred ml of IBV 42 was centrifuged at 600 x g for 30 minutes at 4 C in a PR-1 centrifuge. The supernatant fluid was diluted with 100 ml of PBS. The mixture was again centrifuged at 10,000 x g for one hour at 4 C in a RC-2 Automatic Superspeed Sorvall centrifuge. The supernatant fluid was spun for one hour at 109,000 x g in a Spinco Model L preparative ultracentrifuge at 4 C using a No. 50 rotor. The supernatant fluid was removed. The pellet was suspended in 80 ml of PBS and recentrifuged at 109,000 x g for one hour at 4 C. The pellet was finally suspended in 8 ml of PBS. This twice centrifuged virus was used in density gradient centrifugation and for antigenic analysis.

Density Gradient Centrifugation

Equilibrium density gradient centrifugations (Meselson et al., 1957) were carried out in a Spinco Model L ultracentrifuge at 4 C employing the SW 39 swinging bucket rotor. One ml of the twice sedimented virus sample was carefully layered over 4 ml of 2 M cesium chloride (CsCl) solution in PBS in lusteroid tubes. The control consisted of one ml of PBS substituted for the virus to check the linearity of the gradient. The preparations were then centrifuged at 130,000 x g for 40 hours. The rotor was allowed to decelerate without braking. The bottom of each tube was punctured by a 27 gauge needle and fractions of 4 drops each were collected in 2.7 ml of PBS. Fractions from the control tubes were not diluted. Buoyant densities (ρ) of the control fractions were determined by measuring the refractive index (nd) at 25 C in an Abbé 3 L refractometer. The following equation of Ifft et al. (1961) was used to calculate the buoyant densities.

$$\rho^{(25c)} = 10.8601\ nd^{25} - 13.4974$$

An alternative method of cesium chloride density gradient centrifugation was used in which a nonlinear isodensity gradient was prepared by layering 1.5 ml of CsCl (2.97 M) on 0.7 ml of CsCl (5.94 M) followed by 2.5 ml of the virus. The mixture was spun for 6 hours at 39,000 rpm in SW 39 swinging bucket rotor at 4 C.

Infectivity assays were made by the plaque method. The samples were stored at 4 C until titrated. The virus was not stable in cesium chloride with freezing and thawing.

The antigens from the infected CAM and ether treated virus were also subjected to density gradient centrifugation and were tested by the agar gel diffusion test.

DEAE-Cellulose Chromatography

Fifty grams of DEAE cellulose (0.7 Meq/gram, Cellex D) were mixed with 700 to 800 ml of 0.02 M phosphate buffer, pH 7.2. After the adsorbent had settled, the cloudy supernatant fluid was decanted and was replaced with fresh buffer. The process was repeated until the supernatant fluid was clear. After the last washing, the adsorbent was suspended in sufficient phosphate buffer to make approximately a 60 ml slurry for each gram of dry adsorbent used.

Glass columns of 2 x 20 cm and 1 x 10 cm fitted with fritted discs were used. The larger columns were packed by pouring the slurry to two-thirds the height of the column. When the adsorbent had settled down to a height of about 5 cm, the stopcock at the bottom of the column was partially opened and additional slurry was trickled from an overhead reservoir into the column at a rate governed by the fluid leaving the column. The slurry in the reservoir was mixed gently by means of a magnetic stirrer. After the column was

packed to a height of 25 cm, dry nitrogen at 1 per square inch (p.s.i.) applied momentarily. The pressure was gradually raised to 5 p.s.i. After the small columns were filled with the slurry and the adsorbent had settled, the columns were packed to a height of about 9 to 10 cm by brief application of dry nitrogen at 2 p.s.i. pressure.

The columns were packed at room temperature but were washed overnight with the starting buffer at 4 C prior to use.

Infectious bronchitis virus 42 in allantoic fluid after centrifugation at 600 x g at 4 C was dialyzed against 0.02 M phosphate buffer, pH 7.2, in the cold for 24 hours with 3 changes of buffer.

The virus was added to the columns with a pipette. After the virus had passed through the column, the top of the column was washed with the same quantity of starting buffer as the virus. To elute the virus and its antigens from the adsorbent, 20 ml of 0.02 M phosphate buffer, pH 7.2 containing 0.05 M NaCl was passed through the larger columns. This was repeated until NaCl concentration reached 1 M. The concentration increment at each step was 0.05 M. At a flow rate of 20 ml per hour, 10 fractions of 10 ml each were collected from the larger columns. With the smaller columns, 10 ml of 0.02 M phosphate buffer, pH 7.2 containing different concentrations of NaCl in increments, 0.05 M at each step was passed through. At a flow rate of 10 ml per hour,

fractions of 5 ml each were collected from smaller columns.

All fractions were analysed for protein and were then dialyzed in the cold against PBS for at least 24 hours to remove the excess sodium chloride. The dialyzed samples were passed through a 450 mu Millipore filter to remove any bacteria. One ml of the sample was removed from each fraction for assay of viral infectivity. The fractions were then placed in 10 ml ampoules and lypholized. The samples were reconstituted in one ml of distilled water and tested by the agar gel diffusion using anti-IBV 41 chicken serum.

Neutralization Test

Serum neutralization tests were performed by the method described by Cunningham (1951, 1963a). Undiluted serum was mixed in equal parts with serial 10 fold dilutions of the virus in nutrient broth. Virus controls were prepared by mixing equal volumes of each dilution of virus with nutrient broth. The serum-virus mixtures as well as the virus controls were incubated at 4 C for 30 minutes. Five 10 day old embryonating chicken eggs were inoculated per dilution with 0.1 ml of the sample. Embryo mortality and pathologic changes were the positive responses. The neutralizing index (NI) was the difference between the EID_{50} of virus control and virus-serum mixture.

Growth of IBV and Development of Antigens in the Isolated CAM

The synthesis of precipitating antigens with respect to the synthesis of infective virus was studied in the isolated CAM. Chorioallantoic membranes from 10 day old embryonating chicken eggs were removed aseptically, washed in tris-phosphate-buffered saline (TBS), pH 7.0, (Drake and Lay, 1962) and placed in an Erlenmeyer flask. Twenty ml of IBV 42 (1×10^6 PFU/ml) was allowed to adsorb for one hour at 37 C on a platform shaker at 200 rpm. The unadsorbed virus was poured off and membranes were washed 3 times with 200 ml of TBS. Five ml of low-bicarbonate medium (Drake and Lay, 1962) per membrane was then added and the incubation continued for 42 hours. After 2, 6, 12, 18, 24, 30 and 42 hours, a 2 ml sample was removed and tested for infectivity by the plaque method and for viral antigens by immunodiffusion.

Thermostability of Antigens

The antigens were incubated at 100 C for 10, 30 and 60 minutes and heated samples were tested by immunodiffusion.

Enzyme Sensitivity of Antigens

Enzymes

1. Trypsin: Forty mg of crystalline trypsin (Nutritional Biochemicals Corporation) was dissolved in 10 ml of Tris buffer, pH 8.0, containing 0.01 M magnesium chloride and 0.0025 M $CaCl_2$.

2. Pepsin: Forty mg of crystalline pepsin (Nutritional Biochemicals Corporation) was dissolved in 10 ml of 10% acetic acid.

3. Deoxyribonuclease: One hundred mg of 2 x crystalline DNase (Nutritional Biochemicals Corporation) was dissolved in 25 ml of distilled water containing 0.1% $MgCl_2$.

4. Ribonuclease: Forty mg of 5 x crystalline RNase (Nutritional Biochemicals Corporation) was dissolved in 10 ml of distilled water.

Treatment of Viral Antigens with Enzymes

In this procedure, 0.75 ml of the antigens was treated with 0.25 ml of the different enzymes at a final concentration of 1000 ug per ml. After incubation at 37 C for 12 hours, the mixtures were tested by agar gel diffusion.

RESULTS

Antigens from Viral Allantoic Fluid

Infectious bronchitis virus 41 and 42 in allantoic fluid without further concentration when diffused against homologous chicken serum in agar gel produced one faint line of precipitation. Occasionally 2 lines were produced. The virus allantoic fluid after 10 fold concentration by dialyzing against polyethylene glycol, produced 3 precipitin lines (Figure 1). No precipitin lines were produced by concentrated NAF from 12 day old chicken embryos.

These 3 antigens will be referred to as antigens 1, 2 and 3 based on their position between the antigen and antibody wells. Antigen 1 forms a line nearest the antibody well and the antigen 3 nearest the antigen well. Antigens 1 and 2 form a line very close together. With excess antigen, a single line is present but this can be avoided by changing the concentration of antigens. Precipitation due to antigen 1 appeared as early as 4 hours at room temperature although the time varied depending upon the concentration of the reactants. Six to 12 hours was the optimum period. The precipitation line due to antigen 2 generally appeared after 24 to 30 hours. After about 5 to 6 days, the line due to

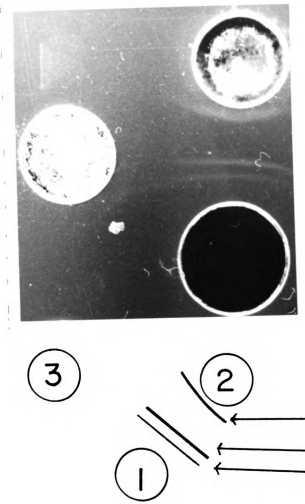


Figure 1. Diffusion of IBV 42 in allantoic fluid and NAF against anti-IBV 41 chicken serum.

1 = Anti-IBV 41 chicken serum
2 = IBV 42 in allantoic fluid
3 = NAF.

antigen 3 appeared near the antigen well. Precipitation was always delayed by at least 24 hours when incubation was at 4 C. The wells were always filled with lower concentrations of reactants than the starting concentrations to avoid duplication of precipitation lines when incubation was for longer than a week.

Differential Centrifugation

Antigens were not sedimented from virus allantoic fluid after one hour at 109,000 x g. The supernatant fluid (Fraction II, Table 1) produced 3 precipitin lines (Figure 2). No precipitation occurred with the resuspended pellet (Fraction VI in Table 1, Figure 2). This could be due to the inability of the virus particles to diffuse into the agar. The virus, after 2 cycles of differential centrifugation at 109,000 x g, was purified 863 fold based on the increase in specific infectivity. There was a 99.81% reduction in protein during purification (Table 1). The results (Table 1) also show that a considerable amount of infective virus remained in the supernatant fluid. This partially purified virus was susceptible to freezing at -60 C.

Although the ultracentrifuged virus did not produce any precipitation in agar gel, it did give a positive ring test against anti-IBV 41 chicken serum.

TABLE 1. Data characterizing the efficiency of differential centrifugation used in the partial purification of IBV 42

	Fraction	Total Volume	Total Infectivity (PFU)	Total Protein (mg)	Specific Infectivity (PFU/mg protein/ml)	Purification Factor[a]	% Reduction in Protein
I	IBV 42-infected-allantoic fluid	100	2.68×10^7	220	1.22×10^5	--	--
II	Supernatant of centrif. at 10,000 x g(diluted 50:50 with PBS)	200	2.24×10^8	232	9.66×10^5	8	0
III	Ist centrif. supernatant (100,000 x g,1hr.)	200	6.24×10^7	120	5.20×10^5	4	45
IV	Ist centrif. resuspended sediment (109,000 x g)	88	9.82×10^7	6.2	1.58×10^7	129	97.2
V	IInd centrif. supernatant (109,000 x g,1hr.)	80	1.34×10^7	2.88	4.70×10^6	39	98.7
VI	IInd centrif. resuspended sediment (109,000 x g)	8	4.56×10^6	.432	1.01×10^8	863	99.81

$$a = \frac{\text{Sp. infectivity of subsequent fraction}}{\text{Sp. infectivity of fraction I}}$$

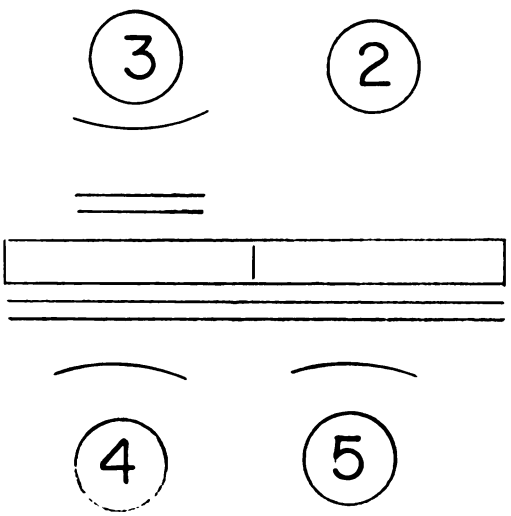


Figure 2. Diffusion of IBV 42 in allantoic fluid, centrifuged virus, supernatant fluid and ether treated virus against anti-IBV 41 chicken serum.

1 = Anti-IBV 41 chicken serum
2 = Ultracentrifuged IBV 42
3 = IBV 42 in allantoic fluid
4 = Supernatant fluid
5 = Ether treated ultracentrifuged IBV 42.

Ether Sensitivity

The IBV is ether sensitive with respect to infectivity. Kinetic studies of inactivation of IBV 42 and IBV 42-110 C by ether showed that the relationship between the surviving virus (log V/V_0) and time is not linear (Figure 3 and 4).

This could be due to either heterogeneity of the virus population or due to mechanistic effects. The data presented here are not sufficient to indicate heterogeneity of the virus. The tailing effects observed could have been due to aggregation of virus particles or adsorption of them to the walls of the vessel or failure of the virus particles to come into contact with the ether due to poor emulsification. The time taken to centrifuge the sample and the subsequent removal of the residual ether by passing dry nitrogen might have affected the results on a basis of time alone.

The results of inactivation of IBV 42 in allantoic fluid by ether at 4 C show that 98.86% of the virus population was inactivated in 5 minutes (Figure 3). In the same period of time, 99% of IBV 42-110 C was inactivated by ether at 4 C (Figure 4). At room temperature, IBV 42 in allantoic fluid was completely inactivated within 5 minutes whereas IBV 42-110 C was inactivated within 15 minutes (Figures 3 and 4).

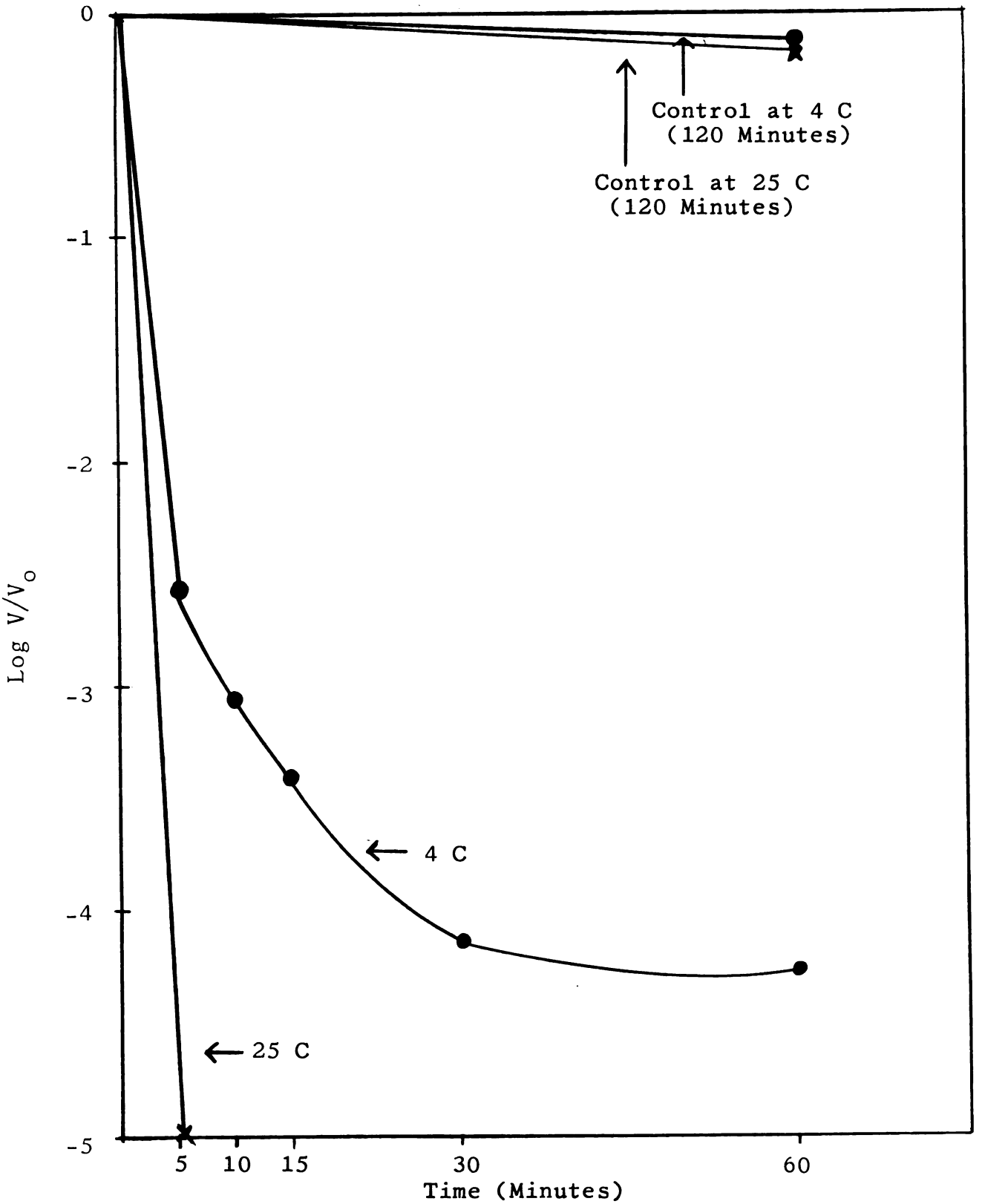


Figure 3. Inactivation of IBV 42 by ether at 4 C and 25 C.

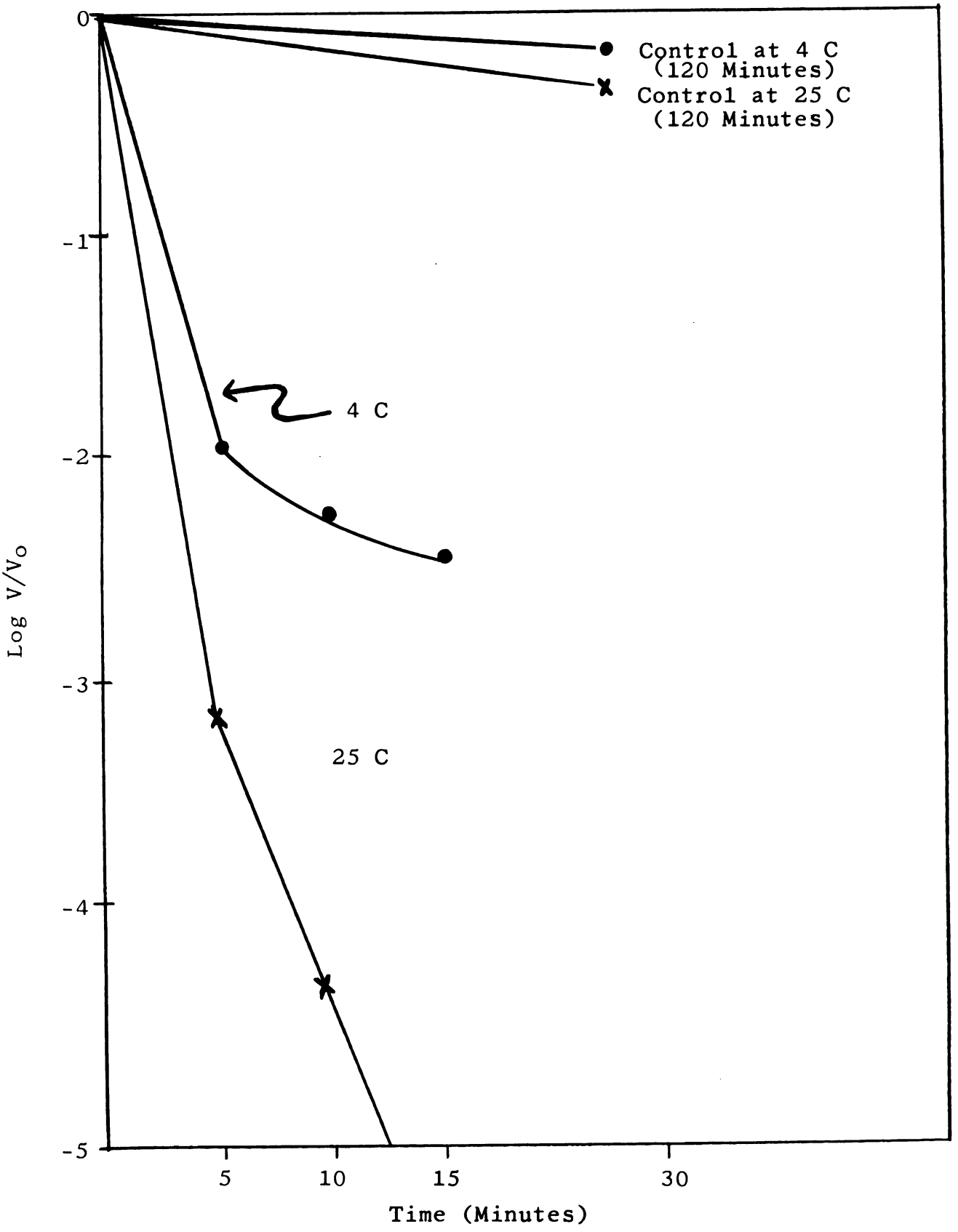


Figure 4. Inactivation of IBV 42-110 C by ether at 4 C and 25 C.

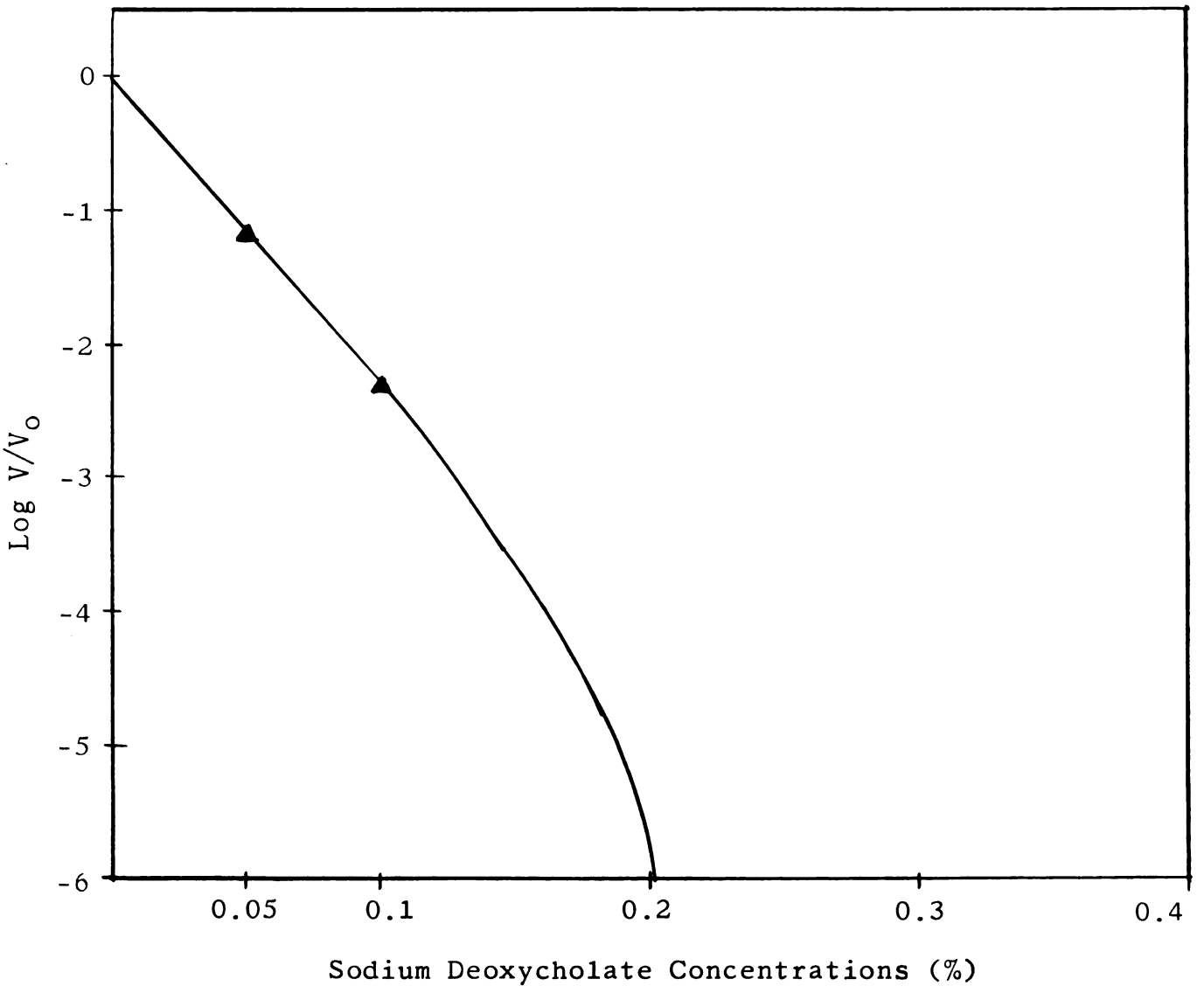


Figure 5. Inactivation of IBV 42 by sodium deoxycholate at 25 C for 10 Minutes.

TABLE 2. Ether inactivation of IBV at 4 C and 25 C

Time of Treatment (Minutes)	4 C Infectivity (PFU/ml)	25 C Infectivity (PFU/ml)
0	1.78×10^6	2.20×10^6
5	4.54×10^3	0.0
10	1.48×10^3	0.0
15	7.05×10^2	0.0
30	1.24×10^2	0.0
60	1.01×10^2	0.0
120	0.0	0.0
120 untreated control	1.73×10^6	2.12×10^6

TABLE 3. Ether inactivation of IBV 42-110 C at 4 C and 25 C

Time of Treatment (Minutes)	4 C Infectivity (PFU/ml)	25 C Infectivity (PFU/ml)
0	6.96×10^5	6.96×10^5
5	7.17×10^3	1.46×10^2
10	3.93×10^3	2.90×10^1
15	9.95×10^2	0.0
30	0.0	0.0
120 untreated control	4.59×10^5	3.30×10^5

*Ether concentration was 2 part virus to one part ether.

Release of Antigens by Ether Treatment

Infectious bronchitis virus is ether sensitive with respect to infectivity but there is the possibility that ether disintegrates the virus into its antigenic components which can be detected by the precipitation test.

When twice ultracentrifuged IBV 42 was treated with ether for 2 hours, 3 antigenic components could be detected in the aqueous and in the interphase but not in the ether phase by the agar gel diffusion. These antigens were identical to those in the concentrated virus (Figure 2). Precipitation appeared earlier than when IBV 42 in allantoic fluid was used. Normal allantoic fluid treated with ether did not precipitate anti-IBV 41 chicken serum.

The IBV 41 after treatment with ether also produced 3 lines of precipitation.

Sensitivity of IBV 42 to Sodium Deoxycholate

The IBV 42 in allantoic fluid was completely inactivated by 0.2% sodium deoxycholate in 10 minutes at room temperature. At 0.5 and 0.1% sodium deoxycholate, 22% and 99.5% of the virus was inactivated respectively (Table 4, Figure 5).

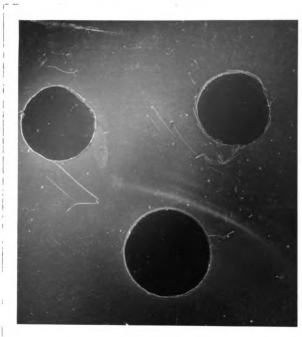
TABLE 4. Inactivation of IBV 42 by sodium deoxycholate at 25 C for 10 minutes

Sodium Deoxycholate Final Concentration (%)	Infectivity (PFU/ml)
0.00	1.10×10^6
0.05	2.40×10^5
0.10	5.70×10^3
0.2*	0.0
0.3*	0.0
0.4*	0.0
0.5*	0.0

*Undiluted samples could not be tested due to the toxicity of sodium deoxycholate to cells.

Antigens from Viral-Infected CAM

Extracts of IBV 42-infected CAM harvested after 20 hours produced 3 lines of precipitation against anti-IBV 41 chicken serum. The 3 lines corresponded to antigens 1, 2 and 3 (Figure 6). The antigen 3 line was nearer to the antibody well than antigen 3 of IBV in allantoic fluid or from ether treated virus. Antigen 3 in CAM preparation could not always be detected.



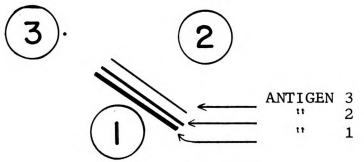


Figure 6. Diffusion of IBV 42 CAM antigens and normal CAM against anti-IBV 41 chicken serum.

1 = Anti-IBV 41 chicken serum
2 = IBV 42 CAM antigens
3 = Normal CAM.

The IBV 41-infected CAM also contained antigens 1, 2 and 3.

When IBV 42-infected CAM preparations were centrifuged at 100,000 x g for one hour, antigen 1 was detected in the supernatant fluid and antigen 2 was detected in the pellet. Antigen 3 was not detected.

Growth of IBV 42 and Development of Antigens in the Isolated CAM

The maximum titer of the virus in the extracellular fluid occurred at 12 hours. The sudden increase of titer of the virus at 6 hours might have been due to the constant shaking of the CAM culture flask on a platform shaker at 200 rpm. The virus was detected in the extracellular fluid up to 42 hours. Antigens 1 and 2 were detected in the extracellular fluid at 6 hours and persisted up to 42 hours (Table 5, Figure 7).

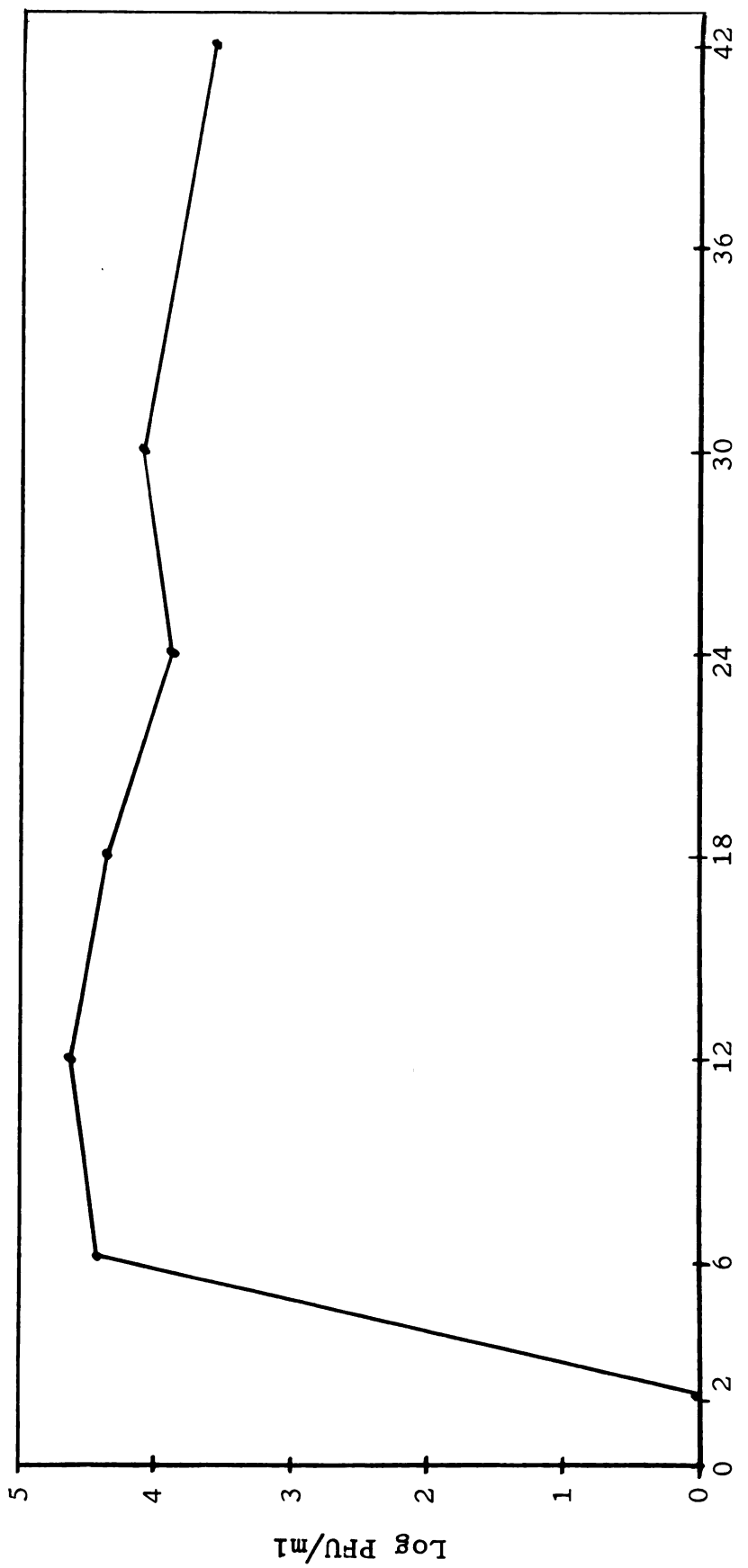


Figure 7. Growth of IBV 42 in isolated chorioallantoic membranes.

TABLE 5. Growth of IBV and development of antigens in isolated CAM on platform shaker

Time (Hours)	Infectivity (PFU/ml)	Antigens Detected		
		1	2	3
0	0.0	-	-	-
2	0.0	-	-	-
6	2.64×10^4	+	+	-
12	3.90×10^4	+	+	-
18	2.41×10^4	+	+	-
24	7.90×10^3	+	+	-
30	1.38×10^4	+	+	-
42	3.99×10^4	+	+	-

+ = present; - = absent.

Relationship Among Antigens from Viral Allantoic Fluid, Ether Treated Virus and Virus-Infected CAM

Antigens 1, 2 and 3 from IBV 42 in allantoic fluid, ether treated IBV 42 and IBV 42-infected CAM were immunologically identical when tested by the agar gel diffusion (Figure 8).

The IBV 42-infected CAM antigens and ether treated virus antigens were diffused from wells 1 and 2, respectively (Figure 9). Twenty-four hours later, wells 1, 2, 6 and 7 were filled with anti-IBV 41 chicken serum whereas wells 3 and 5 were filled with CAM antigens. Well 4 was filled by ether treated virus. Three precipitin lines were produced between antiserum wells 6 and 7 and antigen wells 3, 4 and 5. No line was produced between the antiserum wells 1 and 2 from which CAM antigens and ether treated virus had been previously diffused and antigen wells 3, 4 and 5. The antiserum diffused from wells 1 and 2 was precipitated by the CAM and ether treated virus antigens previously diffused from these wells before filling them with antiserum. The precipitate was in the form of halo. This inhibition technique of Bjorklund (1952) showed that antigens from infected CAM and ether treated virus are immunologically identical.

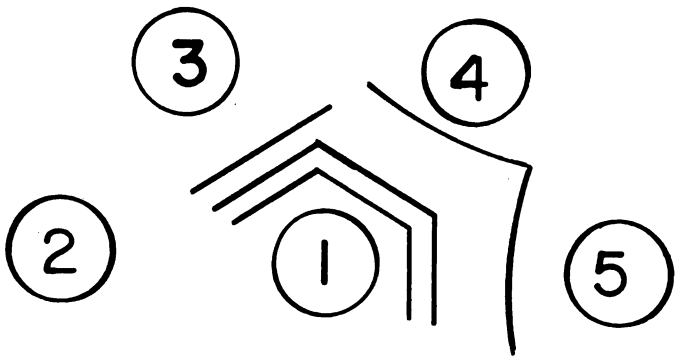


Figure 8. Diffusion of IBV in allantoic fluid, ether treated virus, IBV 42 CAM antigens and NAF against anti-IBV 41 chicken serum.

1 = Anti-IBV 41 chicken serum
2 = NAF
3 = IBV 42 CAM antigens
4 = Ether treated IBV 42
5 = IBV 42 in allantoic fluid.

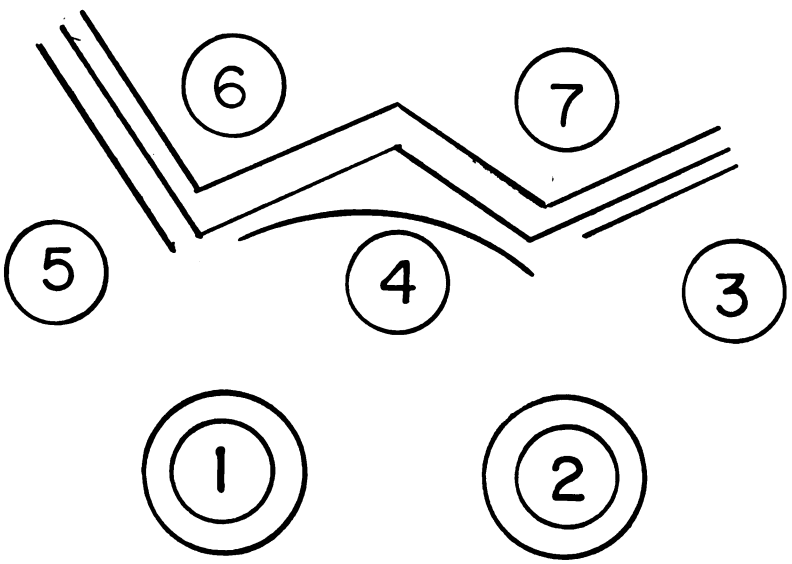


Figure 9. Inhibition technique showing identity between antigens from ether treated IBV 42 and IBV 42-infected CAM.

1 = Anti-IBV 41 chicken serum (IBV 42 CAM antigens were diffused for at least 24 hours before adding the antiserum)

2 = Anti-IBV 41 chicken serum (IBV 42 ether treated virus was diffused for at least 24 hours before adding the antiserum.

3 and 5 = IBV 42 CAM antigens

4 = Ether treated IBV 42

6 and 7 = Anti-IBV 41 chicken serum.

Note the halo around wells 1 and 2 due to the precipitation of antibodies around the wells by the antigens diffused prior to adding antiserum.

Antigenic Relationship Among IBV 41, 42 and 46

Antigens 1, 2 and 3 from IBV 41, 42 and 46-infected CAM and ether treated virus were identical when tested against anti-IBV 41 chicken serum by the agar gel diffusion (Table 6). Antigen 3 of IBV 46-infected CAM could not be detected. The results of cross neutralization test in chicken embryos, however, showed that there are antigenic differences between IBV 41, 42 and 46 that were not detected by the agar gel diffusion (Table 7).

TABLE 6. Relationship between IBV 41, 42 and 46 antigens using agar gel diffusion

Virus Type	Antigen Source	Anti-IBV 41 Serum		
		Antigens Detected		
		Antigen 1	Antigen 2	Antigen 3
IBV 41	CAM	+	+	+
	ETV	+	+	+
IBV 42	CAM	+	+	+
	ETV	+	+	+
IBV 46	CAM	+	+	-
	ETV	+	+	+

+ = Detected; - = could not be detected; and ETV = ether treated virus.

TABLE 7. Neutralization index of anti-IBV 41, 42 and 46 chicken serum

Virus	Anti-IBV Chicken Serum			
	41[a]	41[b]	42	46
IBV 41	5.00	6.32	3.45	4.51
IBV 42	ND	6.70	4.17	3.04
IBV 46	ND	5.19	2.00	5.20
Precipitating antibodies*	+	-	+	-

*+ = positive; - = negative.

[a]Positive for precipitating antibodies.

[b]Negative for precipitating antibodies.

Filtration

Infectious bronchitis virus 42 was not retained by the 450 and 300 mu filter but was retained by the 100 mu filter.

Antigens 1, 2 and 3 from ether treated virus passed through 450 and 300 mu filters. Antigen 3 was retained by the 100 mu filter. Antigen 2 passed through 100 and 50 mu filters but was retained by the 10 mu filter. Antigen 1 was not retained by the 10 mu filter. The antigens from infected CAM passed through 450 and 300 mu filters but were retained by the 100 mu filter (Table 8).

TABLE 8. Filterability of IBV 42 and antigens 1, 2 and 3 from infected CAM and ether treated virus

APD* of Millipore Filter (mu)	Infectivity (PFU/ml)	Antigens in Filtrate	
		CAM Antigens	Ether Treated Virus Antigens
Control	3.1×10^5	1,2,3	1,2,3
450	2.7×10^5	1,2,3	1,2,3
300	1.6×10^5	1,2,3	1,2,3
100	0.0	none	1,2
50	0.0	none	1,2
10	0.0	none	1

*Average pore diameter.

Enzyme Sensitivity of Antigens

On the basis of precipitation, antigens 1 and 2 were sensitive to trypsin and pepsin but DNase had no effect (Figure 10). Antigen 2 was also sensitive to 10% acetic acid and RNase.

Antigen 3 could not be detected in the IBV 42 CAM antigens used as controls and no conclusion can be made as to its sensitivity to the above reagents.

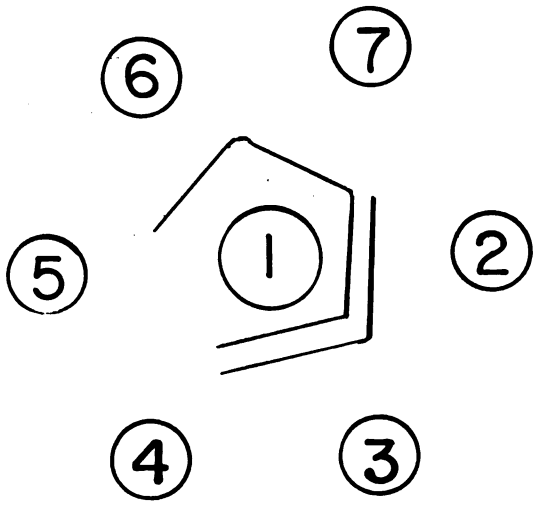


Figure 10. Effect of enzymes on the IBV antigens.

1 = Anti-IBV 41 chicken serum
2 = IBV 42 CAM antigens
3 = Antigens treated with deoxyribonuclease
4 = Antigens treated with trypsin
5 = Antigens treated pepsin
6 = Antigens treated with 10% acetic acid
7 = Antigens treated with ribonuclease.

Antigen 3 in CAM antigens could not be detected in controls.

Thermostability of Antigens

Antigens 1 and 3 were stabile for 30 minutes at 100 C but not for 60 minutes. Antigen 2 was stabile for at least 60 minutes at 100 C (Figure 11).

The intensity of precipitation was increased by heating of IBV 42 CAM antigens at 100 C for 10 and 30 minutes. This indicated that either the antigens were deaggregated or that virus particles were degraded into subunits. To test the latter possibility, the ultracentrifuged IBV 42-110 C which did not precipitate anti-IBV 41 chicken serum in agar gel, was heated at 100 C for 30 minutes and also treated with ether. After heating, 3 antigenic subunits were detected. After treatment with ether only antigens 1 and 2 which were identical with the antigens 1 and 2 from the heated virus were detected. Unheated virus did not produce any lines of precipitation (Figure 12).

Release of Antigens from IBV 42 by Sodium Dodecyl Sulphate

Centrifuged IBV 42, free of soluble antigens, was treated with 1% sodium dodecyl sulphate (SDS) in 0.02 M phosphate buffer, pH 7.2, for 2 hours at room temperature. The mixture was stored at 4 C overnight. The gelatinous precipitation formed by SDS at low temperature was sedimented at 10,000 x g at 4 C. The supernatant fluid was

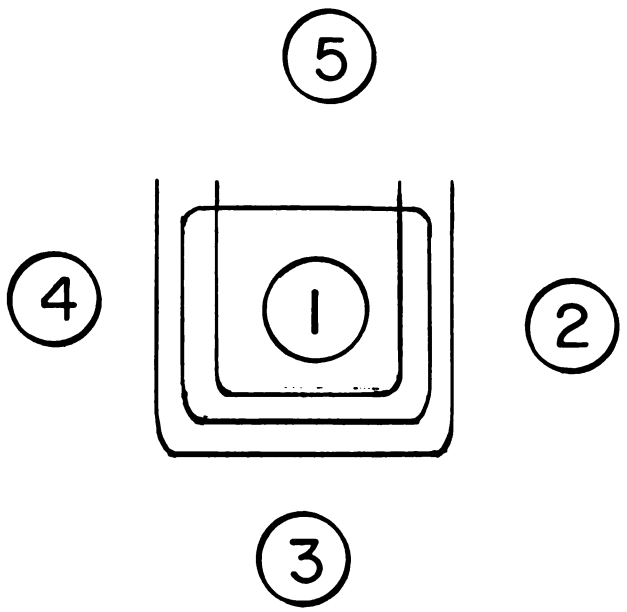


Figure 11. Effect of heat on the precipitating antigens of IBV.

1 = Anti-IBV 41 chicken serum
2 = Unheated IBV 42 CAM antigens
3 = Antigens heated for 10 minutes at 100 C
4 = Antigens heated for 30 minutes at 100 C
5 = Antigens heated for 60 minutes at 100 C.

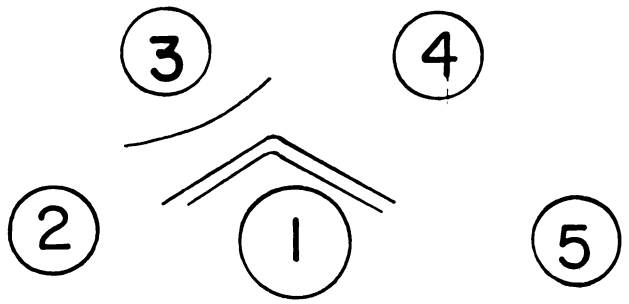


Figure 12. Effect of ether and heat on IBV 42-110 C.

1 = Anti-IBV 41 chicken serum
2 = Unheated ultracentrifuged IBV 42-110 C
3 = IBV 42-110 C heated for 30 minutes at 100 C
4 = IBV 42-110 C treated with ether
5 = Untreated IBV 42-110 C.

tested for viral antigens. Two antigens were released which were identical to the 2 antigens from IBV 42-infected CAM. Antigen 2 was sensitive to RNase.

Role of Soluble Antigens in Virus Neutralization by Specific Antiserum

The purpose of this experiment was to determine if the antigens derived from partially purified IBV 42 by ether treatment compete with the infective virus for the neutralizing antibody which would help in understanding the nature of antibody.

Antigens

A noninfective sample containing 3 precipitating antigens was prepared from partially purified IBV 42 by treatment with ether at room temperature for 2 hours and then was passed through a 450 mu Millipore filter.

Virus

Virus 42-110 C was used since it was free of soluble antigens as previously tested.

All titrations were done in cell culture.

Antiserum

Anti-IBV 41 chicken serum collected 15 days after infection was used. The antiserum was positive for precipitating antibodies and had a neutralizing index of 5.00 in embryonating chicken eggs against IBV 42.

Assay of Neutralizing Activity of Antiserum

The constant serum decreasing virus method using 1/60, 1/120 and 1/240 dilution of serum was employed. In each of 3 series of tubes containing 0.5 ml of the respective diluted serum was added 0.5 ml of PBS. After 30 minutes at 4 C, 0.5 ml of the serial 10 fold dilutions of the virus were added. The mixture was incubated for 30 minutes at 4 C and 0.5 ml of each sample was used to inoculate CEKC.

The controls consisted of a 0.5 ml of virus added to one ml of PBS. After incubation at 4 C for 60 minutes, 10 fold serial dilutions were used as inoculum for CEKC.

Effect of Soluble Antigens on Neutralizing Activity of Antiserum

The procedure employed was the same as that previously discussed except that 0.5 ml of the noninfective sample containing 3 antigens was substituted for the PBS.

Calculation of Neutralizing Index

The following equation (Bradish et al., 1962) was used to calculate the neutralization index (NI) of the undiluted serum (Tables 9 and 10, Figure 13).

$$\log S_o = \frac{1}{n} \cdot \log \frac{V_o}{V} + \log D \qquad (1)$$

where $\log S_o$ = neutralization index

n = slope constant

$\frac{V_o}{V}$ = ratio of residual infectivity to initial infectivity

D = overall dilution of antiserum in the final reaction mixture.

The parameter n was evaluated by the following expression

$$\log n = \frac{\log V_1 - \log V_2}{\log D_1 - \log D_2} \qquad (2)$$

where $\log V_1$ and $\log V_2$ are the residual infectivities after reaction with D_1 and D_2 dilution of antiserum.

On the basis of the results obtained (Tables 9 and 10, Figure 13) the differences in NI are not sufficient to indicate any blocking of the neutralizing capacity of the antiserum by soluble antigens.

TABLE 9. Neutralization index of anti-IBV 41 chicken serum in cell culture

Dilution of Antiserum	Infectivity (Log PFU/ml)	Log V_o/V	Log D	NI for n = 2.97
1/60	2.25	3.75	1.78	3.06
1/120	3.15	2.85	2.08	2.95
1/240	4.04	1.96	2.38	3.05
No serum (Virus control)	6.00	----	----	----

TABLE 10. Neutralization index of anti-IBV 41 chicken serum after treatment with soluble antigens

Dilution of Antiserum	Infectivity (Log PFU/ml)	Log V_o/V	Log D	NI
1/60	2.94	3.06	1.78	2.82
1/120	3.37	2.64	2.08	2.97
1/240	4.27	1.74	2.38	2.97
No serum (Virus control)	6.00	----	----	----

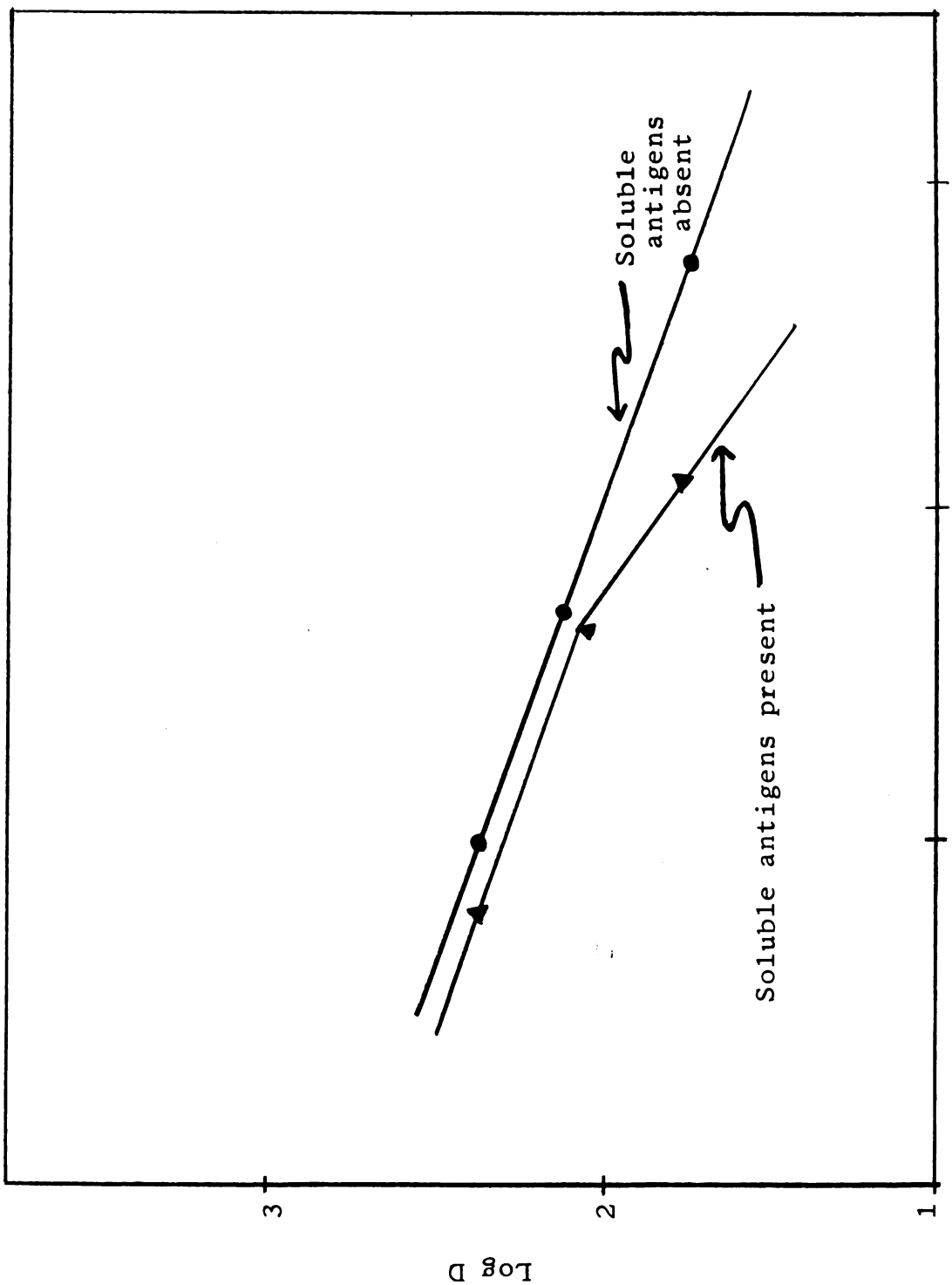


Figure 13. Influence of soluble antigens upon neutralization of IBV 42 C-110 by anti-IBV 41 chicken serum.

DEAE-Cellulose Chromatography

The method used was similar to that described by Philipson (1960). Fifty ml of IBV 42 in allantoic fluid was dialyzed against 0.02 M phosphate buffer, pH 7.2, to equilibrium and was used to load a 2 cm x 20 cm DEAE-cellulose column. The elution was carried out as described under materials and methods. The fractions were analyzed for infectivity, protein and for viral antigens. Approximately 95% of the virus eluted at 0.45 M NaCl whereas about 5% of the infective virus eluted at 0.90 M NaCl (Table 11, Figure 14).

The precipitating antigens were eluted by 0.05 to 0.15 M NaCl. Antigen 1 was eluted by 0.05 M NaCl while antigen 2 was eluted by 0.15 M NaCl. At 0.45 M NaCl, all three antigens 1, 2 and 3 were eluted along with the virus, thus indicating that part of the antigens were bound to the virus.

The behavior of IBV 42 on DEAE-cellulose columns was further investigated with a view to exploring the nature of IBV eluted at 2 different molarities of NaCl. Ten ml of IBV 42 equilibrated with the starting buffer was loaded on a 1 cm x 10 cm DEAE-cellulose column followed by 10 ml of starting buffer. Using the information that the viruses were eluted 0.45 and 0.90 M NaCl, 20 ml of 0.02 M phosphate buffer containing 0.45 M NaCl was passed through the column

TABLE 11. DEAE-cellulose chromatography of IBV 42 in allantoic fluid and its antigens

Fraction Number	NaCl Molarity	PFU/fraction (10 ml/fract.)	% Total PFU	O.D.at 750 mu (Folin)	Antigens Eluted
1	0.05	0	0	0.125	1
2	0.05	0	0	0.120	1
3	0.10	0	0	0.130	1,2
4	0.10	0	0	0.075	1,2
5	0.15	0	0	0.095	2
6	0.15	0	0	0.070	2
7	0.20	0	0	0.020	0
8	0.20	0	0	0.140	0
9	0.25	0	0	1.950	0
10	0.25	0	0	0.410	0
11	0.30	0	0	0.370	0
12	0.30	8×10^2	0.002	0.450	0
13	0.35	1.14×10^4	0.29	0.490	0
14	0.35	ND*	0	0.540	0
15	0.40	ND	0	0.530	0
16	0.40	1×10^5	2.54	0.370	0
17	0.45	1.49×10^6	37.79	0.700	1,2,3
18	0.45	1.66×10^6	42.11	0.420	1,2,3
19	0.50	2.36×10^5	5.99	0.210	0
20	0.50	2.24×10^5	5.68	0.210	0
21	0.55	0	0	0.115	0
22	0.55	0	0	0.075	0
23	0.60	0	0	0.140	0
24	0.60	0	0	0.100	0
25	0.65	0	0	0.075	0
26	0.65	0	0	0.075	0
27	0.70	0	0	0.080	0
28	0.70	0	0	0.040	0
29	0.75	0	0	0.065	0
30	0.75	0	0	0.030	0
31	0.80	0	0	0.030	0
32	0.80	0	0	0.125	0
33	0.85	5.20×10^3	0.13	0.030	0
34	0.85	ND	0	0.070	0
35	0.90	6.60×10^3	0.16	0.045	0
36	0.90	1.78×10^5	4.52	0.100	0
37	0.95	2.60×10^4	0.64	0.120	0
38	0.95	ND	0	0.050	0
39	1.00	3.80×10^3	0.10	0.020	0
40	1.00	0	0	0.040	0

*ND = Not done.

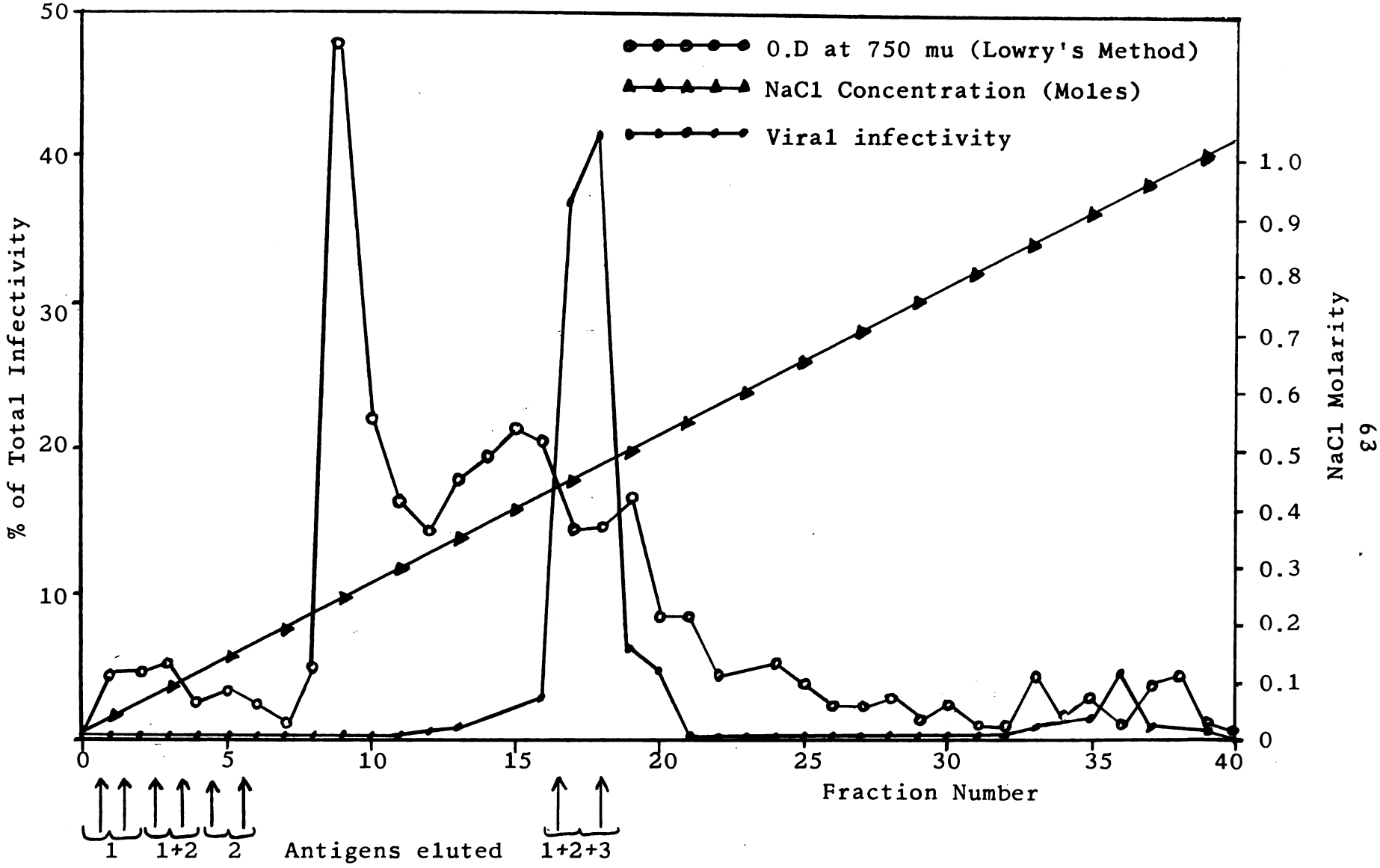


Figure 14. DEAE-cellulose chromatography of IBV 42 in allantoic fluid and its antigens.

and 4 fractions of 5 ml each were collected. The column was then thoroughly washed with 40 ml of 0.02 M phosphate buffer containing 0.45 M NaCl. Thirty ml of the buffer containing 0.90 M NaCl was then passed through the column and 6 fractions of 5 ml each were collected. The fractions were analyzed for infectivity. Approximately 96% of the virus was eluted by 0.45 M NaCl whereas only 4% of the virus was eluted at 0.90 M NaCl (Table 12, Figure 15).

TABLE 12. DEAE-cellulose chromatography of IBV in allantoic fluid

Fraction No.	NaCl Molarity	PFU/fraction	% Total PFU
1	0.45	4.25×10^3	1.09
2	0.45	6.30×10^5	94.98
3	0.45	8.35×10^2	0.12
4	0.45	0.00	0.00
5	0.90	1.25×10^4	1.88
6	0.90	1.02×10^4	1.54
7	0.90	1.69×10^3	0.24
8	0.90	1.35×10^2	0.02
9	0.90	6.70×10^2	0.10
10	0.90	0.00	0.00

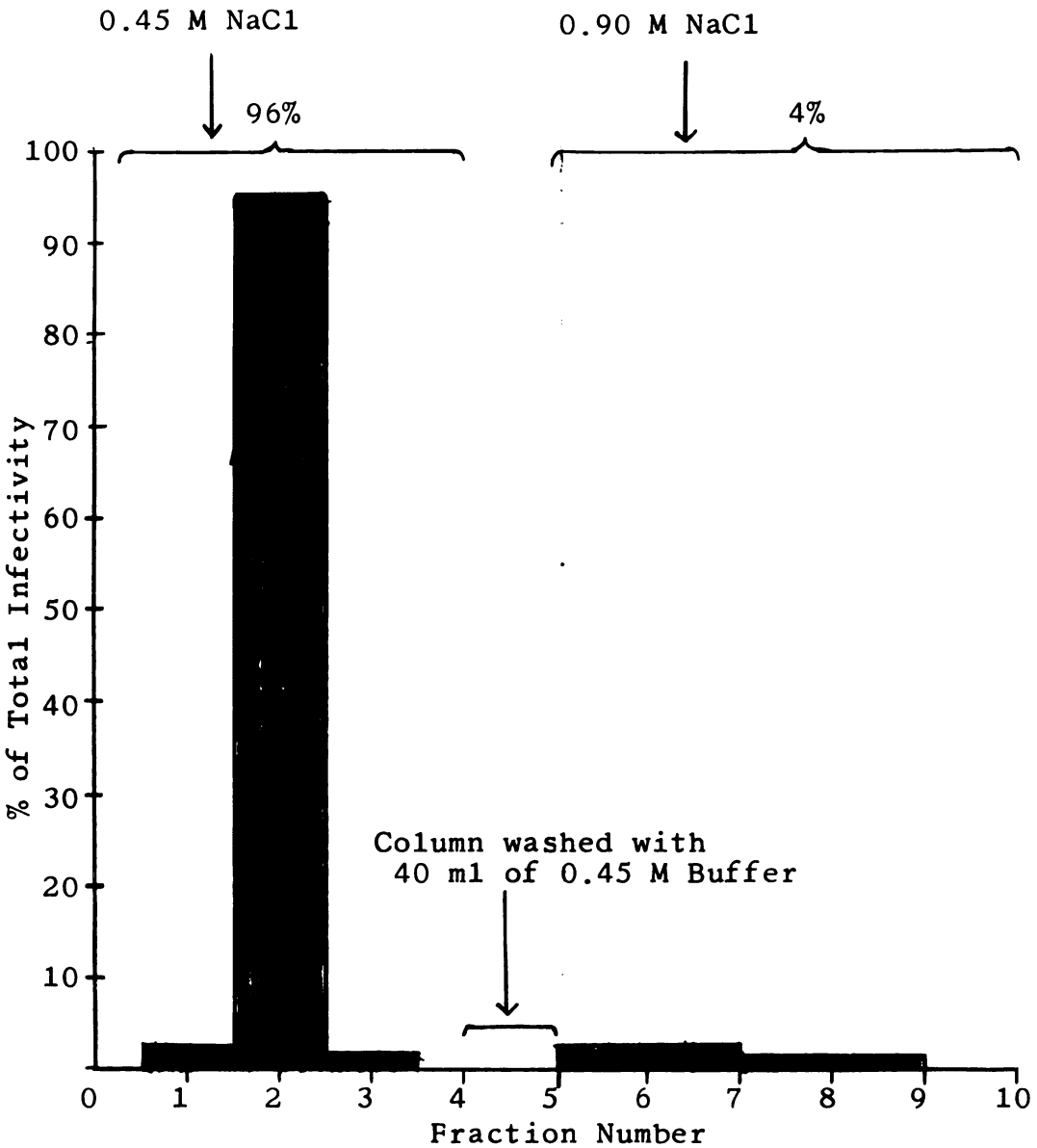


Figure 15. DEAE-cellulose chromatography of IBV 42 in allantoic fluid.

Viruses eluted by 0.45 and 0.90 M NaCl differed in the size of the plaques produced in cell culture. The diameter of 100 plaques from each population was measured using an ocular micrometer in a microscope. The virus eluted by 0.45 M NaCl had a mean plaque diameter of 1.33 mm whereas the virus eluted by 0.90 M NaCl had a mean plaque diameter of 2.89 (Figure 16).

The viruses eluted at these 2 NaCl molarities were specifically neutralized by anti-IBV 41 chicken serum.

Density Gradient Centrifugation

When virus 42 partially purified by 2 cycles of differential centrifugation was examined by cesium chloride density gradient centrifugation, one peak was present (Figure 17). Approximately 54% of the total infectivity of the virus was present in this fraction which had a buoyant density of 1.2289.

Antigens from the 3 different sources were subjected to CsCl density gradient centrifugation. Antigens 1 and 2 could be physically separated with this technique and their buoyant densities detected (Table 13). Antigen from allantoic fluid and ether treated virus had similar buoyant density. The band due to antigen 2 from all 3 sources was broad and it is difficult to derive any definite conclusion

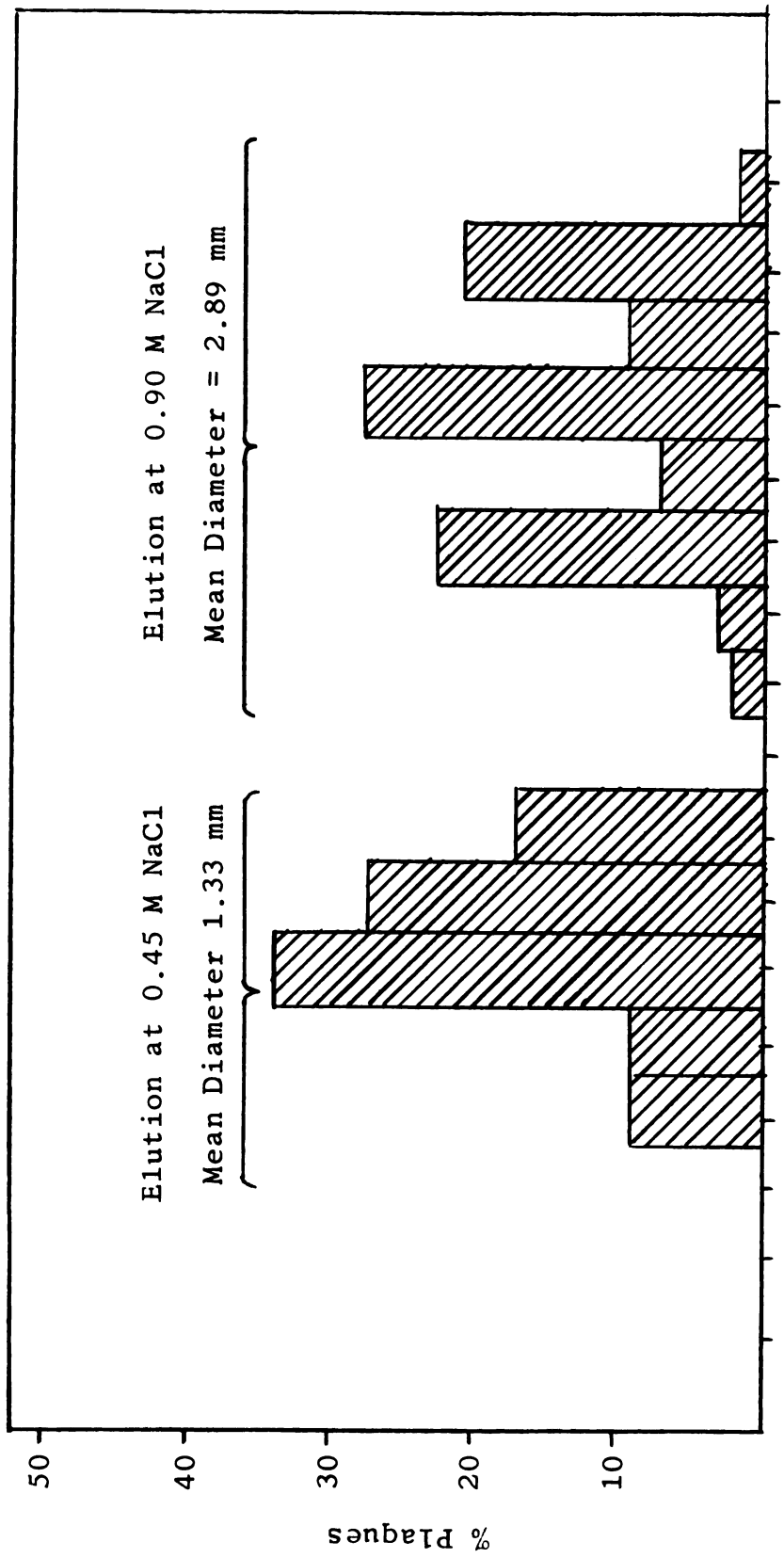


Figure 16. Frequency distribution of plaque diameter of IBV 42 eluted by 0.45 and 0.90 M NaCl.

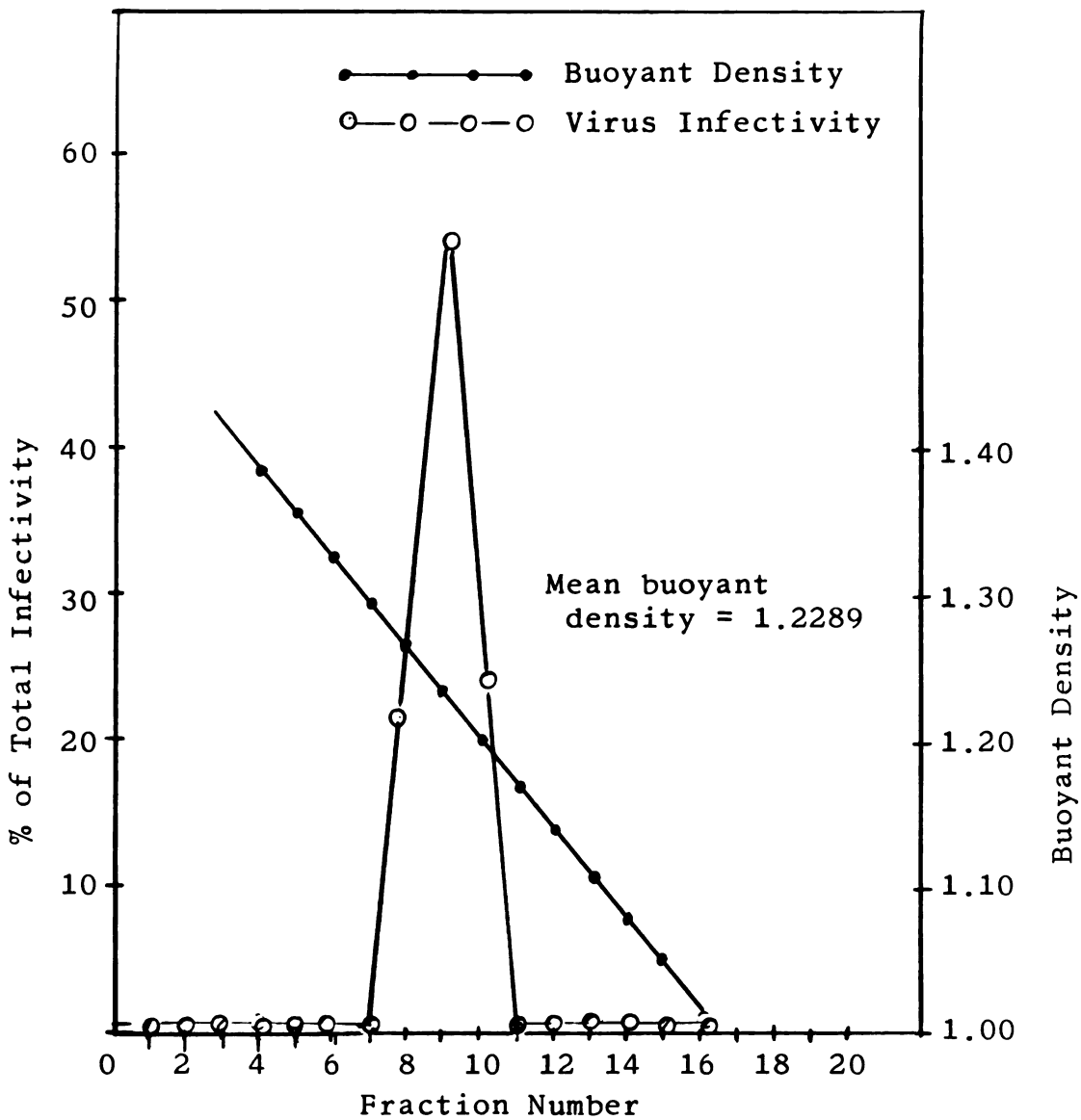


Figure 17. Cesium chloride density gradient centrifugation of IBV 42 twice sedimented at 109,000 X g.

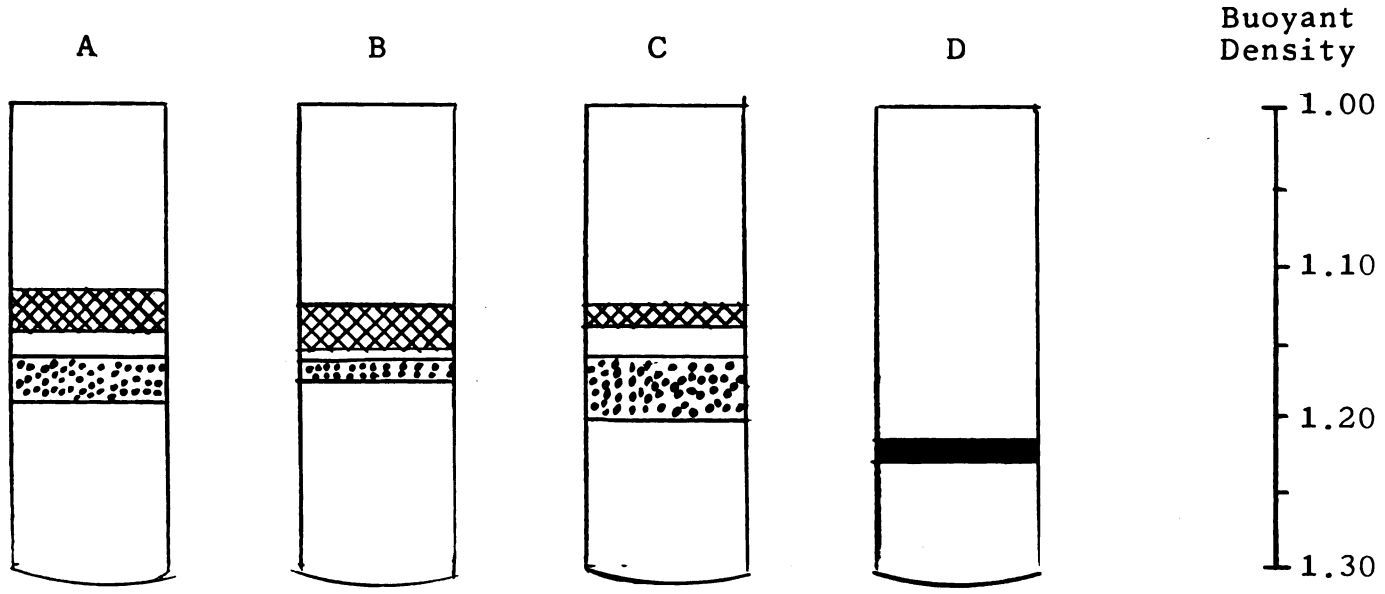


Figure 18. Cesium chloride density gradient centrifugation of IBV 42 and IBV antigens.

A = Antigens of IBV in allantoic fluid
B = Antigens of IBV-infected CAM
C = Antigens of ether treated IBV 42
D = IBV 42.

= Virus = Antigen 1 = Antigen 2

as to the specificities and meaning of the mean buoyant density data. All antigens had a lower density than that of 1.23 of the virus.

TABLE 13. Comparison of mean buoyant densities of viral antigens from infected CAM, viral allantoic fluid and ether treated virus

	Antigen Source		
Antigen Number	Allantoic Fluid	CAM	Ether Treated Virus
1	1.1351	1.1443	1.1360
2	1.1814	1.1760	1.1717
3	*	*	*

Buoyant density of IBV 42 in allantoic fluid was 1.2289.

*Could not be determined due to insufficient concentration of antigen.

DISCUSSION

Chicken serum behaves differently in precipitation reactions than does mammalian serum. The optimum conditions established for precipitation tests in agar gel with IBV and anti-IBV chicken serum are in agreement with the finding of Goodman et al. (1951) that 8% NaCl is an absolute requirement for optimum precipitation of antigen by the antibody. Enhancement of precipitation of the virus and antiserum when normal chicken serum is incorporated in the diffusion medium is not very surprising since it has been shown (Dardas, 1963) that certain cofactors of normal chicken serum contribute towards the bulk of the immune precipitate. It would be interesting to investigate the nature of these cofactors and to determine if they have any influence on neutralization of viral infectivity.

The interference by merthiolate with the immunodiffusion reaction of poliovirus has been reported by Le Bouvier (1957). Similar results were obtained with IBV. The interference could be due to the binding of heavy metal by the antigens in a way as to make the antigens unstable. As a result of this, nonspecific precipitation may occur and thus cause difficulty in reading specific lines of precipitation.

The preliminary investigation to develop a standard technique established that there are 3 antigens, 1, 2 and 3, in IBV in allantoic fluid when tested against anti-IBV chicken serum. Only 2 antigens in the viral allantoic fluid were demonstrated when antiserum was prepared in rabbits (Tevethia, 1962). Witter (1962), on the other hand, demonstrated only one antigen using anti-IBV chicken serum, but the technique employed was not as precise as that developed in the present study.

The failure to sediment the 3 antigens in IBV allantoic fluid after one hour at 109,000 x g indicates their soluble nature since the infective virus was sedimented under the same conditions. There are 2 possibilities with regards to the nature of these antigens: (1) the 3 antigens are altered proteins synthesized by the cells as a result of virus infection, or (2) they are virus subunits that are synthesized by the virus-infected cells in large excess during virus multiplication and are released into the allantoic fluid. Antigens in the IBV-infected CAM are immunologically identical to the antigens in viral allantoic fluid. The release of the antigens into the extracellular fluid from IBV-infected CAM cultivated *in vitro* parallels the release of infective virus. This indicates that antigens in the CAM and in the viral allantoic fluid are related to the virus. Antigen 2 from the infected CAM is sedimentable at 109,000 x g in one hour. This might be due to a firm association of

these antigens with the cellular material since after one hour of sonification at 15 KC did not completely remove the antigens from the cellular material.

Sensitivity of IBV to ether and sodium deoxycholate indicates the presence of peripheral lipids in the virus. The virus is also sensitive to trypsin (Muldoon, 1960). The size of the virus is reduced upon trypsin treatment (Nazerian, 1960). This indicates that the virus coat or envelope is made of lipo-protein. Removal of these peripheral lipids results in the degradation of viral particles accompanied by the loss of infectivity and release of 3 viral antigens. The release of soluble antigen and of the hemagglutinin on treatment of the virus with ether has been reported with influenza virus (Hoyle, 1952; Lief and Henle, 1956). That the 3 antigens released from purified virus by ether are identical immunologically to the 3 antigens in IBV-infected CAM and in viral allantoic fluid indicates that antigens from these sources are components of the virus. This finding is supported by the results obtained by Wilcox and Ginsberg (1963b) with adenovirus. They demonstrated that structural units of adenovirus obtained by disrupting the purified virus by dialyzing at pH 10.5, were antigenically similar to the soluble antigens which accumulated in the adenovirus-infected cells. In the case of Newcastle disease virus, the inner nucleoprotein component of the

virus is identical to the soluble antigen extracted from infected cells (Rott et al., 1963).

Bjorklund's inhibition technique provides further proof of the identity of the antigens from infected CAM and ether treated virus. The CAM antigens absorbed all antibodies against ether treated virus antigens and vice versa thereby showing the relationship.

Treatment of partially purified IBV with sodium dodecyl sulphate results in the release of 2 antigens. One of the 2 antigens is sensitive to RNase. The release of the antigens may be due to the combination of SDS with the positively charged residues on the virus protein coat. Sodium dodecyl sulphate can solubilize poliovirus protein under acid conditions (Mandel, 1964). Maizel (1964) reported the degradation of adenovirus by SDS into 9 protein components which were detected by acrylamide gel electrophoresis.

The sensitivity of antigen 1 to trypsin and pepsin indicates its protein nature. Antigen 2 is a ribonucleoprotein since it is sensitive to RNase, trypsin and pepsin. This antigen has properties similar to the soluble antigen of influenza virus which is sensitive to trypsin and RNase (Hoyle, 1952). This antigen probably represents the internal component of the virus.

Filtration studies indicate that the viral antigens are smaller in size than the virus. Antigen 1 is smaller than antigen 2 and antigen 3 is larger than antigen 1 and 2. The failure of the CAM antigens to pass through a 100 mu filter could be due to their association with the cellular material. This is possible since IBV belongs to the class of viruses which matures at the cellular periphery and probably derives its lipid coat from the host cell. According to Franklin (1962), if the ratio of the released virus to cell associated virus is less than 1, then the virus is usually formed at the periphery. For IBV this ratio has been shown to be less than 1 (Akers, 1963).

The antigens of IBV can be separated on DEAE-cellulose. Antigen 1 elutes at 0.05 M NaCl, whereas antigen 2 elutes at 0.15 M NaCl. The virus elutes at 0.45 and 0.90 M NaCl. The difference in the elutability of the antigens and of the virus at different molarities of NaCl indicates the differences in charged groups on the virus and the antigens. The elutability of the infective virus at 0.45 and 0.90 M NaCl may be due to the presence of 2 populations of virus differing in their affinities towards DEAE-cellulose. The differences in the size of the plaques produced by virus eluted by 0.45 and 0.90 M NaCl supports this view.

The density of antigens 1 and 2 are 1.13 and 1.17, respectively, which is lower than the virus density of 1.23.

The density of IBV in allantoic fluid is similar to the density of NDV, 1.22, in allantoic fluid (Stenback and Durand, 1963).

The precipitation test is not sensitive enough to detect antigenic differences between IBV 41, 42 and 46. It is clear from the cross neutralization test that antigenic differences do exist among IBV 41, 42 and 46. These studies do indicate, however, that the precipitation is highly specific for IBV and even the antigens from viral allantoic fluid are specific.

Precipitating antibodies against IBV appear as early as 7 days and generally disappear after 20 to 25 days. There are 3 possibilities as to the nature of precipitating antibodies: (1) precipitating antibodies are different than neutralizing antibodies, (2) precipitating antibody sites are on the same antibody molecule carrying neutralizing antibody sites, and (3) precipitating antibody sites are the same as neutralizing sites. The third possibility can be entirely ruled out since soluble antigens do not block the neutralizing capacity of the serum, indicating that the sites are different. The second possibility can also be eliminated since precipitating antibodies decrease as the neutralizing antibodies increase. If the sites would have been on the same antibody molecule then precipitating antibodies should persist as long as the neutralizing antibodies.

The first possibility seems to be the most logical one since precipitating antibodies appear earlier than neutralizing antibodies and also disappear soon after the neutralizing antibodies start to rise. The failure of soluble antigens to block the neutralizing capacity of the serum supports this view. At a 1/60 dilution of serum there is a 0.14 log depression of the neutralization index but this could be due to the trapping of neutralizing antibody molecules by the soluble complexes formed by the serum and soluble antigens. The log depression of the neutralization index of the serum is not significant enough to conclude that there is a blocking of the neutralizing capacity of antiserum.

The results obtained do help in understanding the location of antigens on the virus particle. The positive ring test with the centrifuged virus indicates the antigen or antigens are on the surface of the virus particles. Franklin (1962) suggested the possibility that in the case of viruses possessing a lipoprotein coat, the antigens can express themselves if the membrane is incomplete and the antigens are exposed at the surface. This might apply to IBV. Recently Berry et al. (1964) demonstrated that IBV particles possess spikes on the surface. The spikes are attached to the virus surface by a narrow neck and have a bulbous mass of 9 to 11 mu diameter at the distal ends.

The virus appears to contain a component similar to Myxoviruses. The virus also possesses a lipoprotein coat. It is possible that antigen 2 of IBV, which is a ribonucleoprotein, represents the internal component and is released from the virus by ether, sodium dodecyl sulphate and even on heating. The position of antigen 1 is questionable but it could represent the component in the spikes since the spikes could also account for the positive ring test given by the centrifuged virus. Infectious bronchitis virus agglutinates chicken erythrocytes after trypsin treatment and it is possible that spikes on the virus surface might carry the hemagglutinating activity similar to Myxoviruses. That IBV possesses a true hemagglutinin is indicated by the observation that trypsin treated virus reduces the electrophoretic mobility of chicken erythrocytes by 21% compared to 41.9% reduction by influenza virus. Receptor destroying enzyme removed all the receptors from the surface of the erythrocytes and there was no change in the electrophoretic mobility of these erythrocytes upon treatment with trypsin-modified IBV or influenza virus (Biswal, 1963).

Andrews (1964) made no attempt to classify IBV. Data collected might be useful in justifying a place for IBV in virus classification. Hsiung (1964) proposed a classification of viruses based on the size, type of nucleic acid and sensitivity to ether. According to size, the viruses can be grouped into large, medium and small. Large

viruses are those which can not pass through 100 mu filter, medium size are those which can pass through 100 mu but not through 50 mu filter and small viruses can pass through 100 and 50 mu filters. Using Hsiung's scheme of classification, IBV falls under Myxoviruses since it is retained by a 100 mu filter, contains RNA and is ether sensitive.

Using the classification proposed by Lwoff et al. (1962), IBV should be included under Myxoviruses as it is a RNA virus, the capsid is enveloped, and has a size of 80 to 120 mu.

The following properties of IBV are similar to those of the Myxovirus group and would indicate the inclusion of IBV in the Myxovirus group.

1. The virus forms syncytia in cell culture.
2. The virus is formed at the periphery of the cytoplasm.
3. The internal component of IBV is a ribonucleoprotein released by ether.
4. The virus has projections at the surface.

Although the properties of IBV cited above justify its inclusion in the Myxovirus group, further work is needed with regards to the presence or absence of a true hemagglutinin, the biological role played by the projections on the virus surface, and the detailed study of the structure of internal component which is RNase sensitive.

SUMMARY

1. Infectious bronchitis virus in allantoic fluid contains 3 antigens which can be demonstrated by the immunodiffusion test. Antigens 1, 2 and 3 can also be isolated from the IBV-infected CAM and are identical to the antigens from virus allantoic fluid.

2. Infectious bronchitis virus 42 in allantoic fluid is completely inactivated by 33% ether in 5 minutes at room temperature, whereas IBV 42-110 C from CEK cultures is inactivated within 15 minutes at room temperature.

3. The virus is completely inactivated by 0.02% sodium deoxycholate within 10 minutes at room temperature.

4. Treatment of partially purified IBV with ether releases 3 viral antigens which are identical to those of the virus in allantoic fluid and the infected CAM indicating their common origin.

5. Soluble antigens from virus in allantoic fluid are not sedimented after one hour at 109,000 x g. The virus is sedimented under the same conditions.

6. Antigen 1 from ether treated IBV passes through a 10 mu Millipore filter whereas antigen 2 passes through a 50 mu filter but is retained by a 10 mu filter. Antigen 3

passes through a 300 mu filter but is retained by a 100 mu filter. The virus particle is retained by a 100 mu filter but passes through a 300 mu filter.

7. The infectious virus and the soluble antigens can be detected in the extracellular fluid of CAM cultivated in vitro 6 hours after infection.

8. Heating of partially purified IBV at 100 C for 30 minutes releases antigens 1, 2 and 3. Treatment of the virus with sodium dodecyl sulphate releases 2 antigens, one of which is sensitive to RNase.

9. Antigen 2 is a ribonucleoprotein and is sensitive to RNase, trypsin and pepsin but is resistant to DNase. Antigen 1 is sensitive to trypsin and pepsin but is resistant to RNase and DNase. Antigens 1 and 3 are destroyed by heating at 100 C for 60 minutes whereas antigen 2 is resistant to heating at 100 C for 60 minutes.

10. Antigenic differences between IBV 41, 42 and 46 can not be detected by the immunodiffusion technique, but can be by cross neutralization tests.

11. The antigens can be separated on DEAE-cellulose. Antigen 1 and 2 are eluted at 0.05 M and 0.15 M NaCl in 0.02 M phosphate buffer at pH 7.2, respectively. About 96% of the infective virus elutes at 0.45 M NaCl whereas only 4% of the virus elutes at 0.90 M NaCl.

12. Antigens 1 and 2 have a buoyant density of 1.13 and 1.17, respectively. The 2 antigens can be separated from each other by cesium chloride density gradient centrifugation. The virus in allantoic fluid has a buoyant density of 1.23.

13. The noninfective soluble antigens do not block the neutralizating capacity of anti-IBV 41 chicken serum indicating that probably precipitating and neutralizing antibodies are different. The precipitating antibodies are detectable as early as 7 days postinoculation but not 25 days after infection.

14. On the basis that IBV is (1) sensitive to ether and sodium deoxycholate, (2) has a ribonucleoprotein internal component sensitive to RNase, (3) has a lipoprotein envelope with surface projections like those of influenza virus, (4) forms syncytia in cell culture, (5) is an RNA virus and (6) matures at the periphery, it may possibly be considered as a member of Myxovirus group.

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