ANTIGENIC ANALYSIS OF INFECTIOUS BRONCHITIS VIRUS BY AGAR GEL DIFFUSION

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ANTIGENIC ANALYSIS OF INFECTIOUS BRONCHITIS VIRUS BY AGAR GEL DIFFUSION

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INTRODUCTION

Neutralization is the only test used for determination of antigenic relationships among the types of infectious bronchitis virus. Since this test does not give information as to the number and nature of the antigens, the agar gel diffusion test was used in the present study of infectious bronchitis virus.

The objectives of the present investigation were: (1) to study the antigens of IBV in infected allantoic fluid, and (2) to study the antigenic relationship among certain types of infectious bronchitis virus.

LITERATURE REVIEW

Infectious Bronchitis Virus

Infectious bronchitis virus (IBV), the causative agent of infectious bronchitis of chickens, is classified as Tarpeia pulli in Order Virales (Merchant and Packer, 1961; Van Rooyn, 1954). The virus is a sphere with a diameter of 65 to 135 mu (Reagan et al., 1950; Reagan and Brueckner, 1953; Nazerian, 1960).

The virus can be readily cultivated in the allantoic cavity, amnionic cavity, and chorioallantoic membrane of chicken embryos. Serial passage of the virus in chicken embryos decreases the virulence and immunogenicity of the virus for chickens. Completely embryo adapted virus kills all embryos within 24 to 30 hours (Cunningham, 1957).

The Beaudette embryo adapted strain can be easily cultivated in the isolated chorioallantoic membrane (Ferguson, 1958; Ozawa, 1959), chicken embryo kidney cells (Spring, 1960), chicken liver and heart cells (Fahey and Crawley, 1956), and chicken embryo fibroblasts (Spring, 1960).

Chicken red blood cells are agglutinated by trypsin modified IBV only. Trypsin modified virus heated at 56 C for 6 hours retains its hemagglutinating activity. Viral

infectivity is reduced on treatment with trypsin (Corbo and Cunningham, 1959; Muldoon, 1960).

The virus exists in two phases; the thermostable original (O) phase as originally isolated from chickens and the thermolabile derivative (D) phase derived through serial passage in chicken embryos. Only the O phase is antigenic and pathogenic to chickens (Singh, 1960).

Virulent IBV produces antibodies in chickens which can be detected by the neutralization test two weeks after the primary stimulus. Antigenicity of IBV and production of neutralizing antibodies is directly related to infectivity of the virus for chickens (Cunningham, 1957).

Immunological differences among types of IBV are detectable by reciprocal neutralization tests (Jungherr et al., 1956; Hoftad, 1958). Two antigenic types of IBV, Massachusetts and Connecticut, are generally recognized. The Massachusetts type induces the formation of antibodies that are readily detected with the Beaudette and Massachusetts antigens. The Connecticut type induces the formation of antibodies that are not detected by either Massachusetts or Beaudette type (Cunningham, 1960b).

Neutralization of Connecticut antigen by anti-Massachusetts chicken serum is related to the embryo passage level of the antigen. Neutralization does not occur with low embryo passage virus but does occur with higher embryo passage virus (Oshel, 1961).

Diffusion and Precipitation

The reaction of antigen and antibody results in the formation of a precipitate through a two stage reaction. The first stage is a rapid, invisible combination of antigen and antibody molecules to form a specific but reversible complex. The second stage or aggregation stage, starts with the formation of lattices visible as antigen-antibody complexes (Crowle, 1961).

The specific precipitation is affected by certain physico-chemical factors among which are the amount of electrolyte present (Aladjem and Lieberman, 1952), temperature (Boyd, 1956), pH (Graber, 1959), and other factors such as proportions in which antigen and antibody are used, and presence of lipids and proteins foreign to the reactants. Maximum precipitation will be obtained in minimum time when both reactants are used in the region of optimum proportions (Crowle, 1961).

The basic principles of precipitation are governed by the laws which govern diffusion. Substances diffuse because the molecules are in a constant state of agitation due to their thermal energy. There is an over-all tendency for entropy to increase with the result that there is a movement of molecules from an area of higher concentration to one of lower concentration (Randall, 1958). The diffusion rate is affected by such factors as the initial concentration of the substance, temperature, gravitational field, presence of

non-specific substances and the size and shape of the diffusing molecules. The diffusion rate of a substance is directly related to the initial concentration and temperature but indirectly related to the molecular weight (Crowle, 1961). The distance that a substance diffuses from a given boundary is directly proportional to the square root of time (Ouchterlony, 1958).

Precipitating systems may be classified as follows (Ouchterlony, 1958; Oudin, 1952): Simple, in which a single antigen reacts with an antibody; Complex, in which several antigens cross react with a given antibody; and Multiple, consisting of antigens and antibodies reacting simultaneously which may be components of several simple and complex precipitating systems.

Diffusion in semisolid media can be classified as simple and double. Simple diffusion in which one of the reactants diffuses actively into the stabilizing medium containing the other, was first utilized by Oudin (1946) for antigenic analysis of normal horse serum. Double diffusion, in which both reactants diffuse towards each other through a semisolid medium, was utilized by Ouchterlony (1948) and Elek (1948).

The double diffusion test is useful in resolving multiple precipitating systems into their individual components and to compare two antigens against the same antiserum or two antibodies against the same antigen. In a

simple precipitating system containing a single component, the antigen and the corresponding antibody, after diffusing towards each other in agar gel, will combine at equivalent concentrations to form a visible precipitation band. The resolution of a multiple precipitating system into individual components is based on the assumption that specific precipitates in semisolid media permit the diffusion of unrelated antigens and antibodies. The number of precipitation lines obtained in a multiple precipitating system represents the minimum number of antigenic components present although this may not be the maximum number (Ouchterlony, 1958). If two identical antigens are compared by the double diffusion technique, fusion of the precipitation lines due to individual components to form a continuous band or arc has been termed "Reaction of Identity." Diffusion of two serologically related antigens against an immune serum containing specific antibodies gives a precipitation pattern, termed "Reaction of Partial Identity." The pattern consists of a continuous arc with a spur like extension above the arc extending from the precipitation line and is due to the homologous antigenic component. When two serologically unrelated antigens are diffused against an immune serum containing homologous antibodies, the precipitation lines formed, due to unrelated antigenic components, will cross each other. This type of reaction pattern is termed "Reaction of Non-Identity" (Ouchterlony, 1958).

Bjorklund (1952) modified Ouchterlony's double diffusion plate technique, the so-called inhibition plate technique, for analysis of diphtheria and tetanus toxins. The technique was based on the principle that on pretreatment of the diffusion medium with a sufficient amount of a component of a complex immunologic system, the subsequent appearance in the medium of precipitates, corresponding to this particular component, could be completely inhibited. Diffusion of the other components of the same system and the formation of other immunoprecipitates was not influenced.

Grasset et al., (1956) developed a micro-gel diffusion technique in order to compensate for the large volumes of reactants needed in Ouchterlony's double diffusion plate technique. The micro-gel diffusion test has been modified and used successfully by Mansi (1958), Crowle (1958), and Murty (1960), for analysis of various antigenic mixtures.

Application of Agar Gel Diffusion Text in Virology

Jensen and Francis (1953) were the first to use the agar diffusion test in virology. The antigenic components of PR8, Rhodes, and Lee strains of influenza virus could be distinguished. Multiple lines of precipitation indicated the presence of more than one antigen.

Gispen (1955) analyzed pox virus antigens using the agar gel diffusion technique of Oakley and Fulthorpe (1953) in tubes. The diffusion pattern of vaccinia virus against

anti-vaccina rabbit serum was composed of 3 to 6 zones of specific precipitation due to soluble antigens. There was no essential difference among the precipitation patterns of vaccinia, neurovaccinia, rabbit pox, and variola viruses when diffused against anti-vaccinia rabbit serum. Fraction I of cow pox virus had the same diffusion properties as fraction I of vaccinia virus. These two fractions could be differentiated antigenically using liquid media for the precipitation test.

Wittmann (1958) demonstrated antibodies against fowl pox virus by the double diffusion test using anti-fowlpox chicken serum. The ability of the chicken to form precipitins was directly related to the age of the chicken. Virulent strains of the virus were the most effective for stimulation of antibody production.

Forsek (1958) employed the pancreas from pigs experimentally infected with swine fever virus, as antigen and found the precipitation test to be specific for demonstration of immunity in vaccinated pigs. Grishenkova (1958) used saline suspensions of lymph nodes or blood from swine fever virus infected pigs as the antigens and demonstrated specific precipitation with serum from infected or immune pigs and rabbits.

Bodon (1955) demonstrated single lines of precipitation with types O, A, and C of foot and mouth disease virus (FMDV) with anti-FMDV bovine serum. This work was confirmed by

Brown and Crick (1957), but in addition it was found that any of the types produced two distinct lines of precipitation with homologous antiserum. Brown and Crick (1958) demonstrated two lines of precipitation when virus in the form of vesicular fluid from guinea pigs infected with FMDV was diffused with its homologous antiserum. These two lines corresponded to the individual lines produced with 20 mu and 7 mu particles of FMDV obtained by differential ultracentrifugation, when they were diffused separately with the same antiserum. After heating the virus for 30 minutes at 56 C, only one line of precipitation, due to the 7 mu component, was produced. The 20 mu component was considered to be type specific.

Le Bouvier (1957) demonstrated that when poliomyelitis virus types I, II, and III contained in extra-cellular culture fluid were diffused against homologous hyperimmune mokey serum, major and minor lines of precipitation were produced. Le Bouvier et al., (1957) demonstrated that the major line of precipitation was produced by the fraction of poliomyelitis virus containing infective particles with 20 to 30 per cent ribonucleic acid (RNA). The minor line was produced by the fraction containing non-infective virus with very little RNA. Grasset et al., (1958) using poliomyelitis virus concentrated by ultracentrifugation from extracellular culture fluid demonstrated two lines of precipitation with anti-poliomyelitis virus type I and type II monkey serum but

only one line with type III. Antiserum from human beings vaccinated with Salk vaccine produced lines of precipitation with types I, II, and III.

According to White (1958), only one line of precipitation occurred with rinderpest virus-infected lymph nodes from cattle and anti-rinderpest virus rabbit serum. Antigens prepared from rinderpest virus-infected lymph nodes of goats and cattle were identical. Stone (1960) used rinderpest virus infected mesenteric lymph nodes of cattle as antigen and demonstrated at least two lines of precipitation. The faster moving component was heat labile and the slower moving component was heat stable.

Using the micro-gel diffusion test with duck hepatitis virus-infected chicken embryo liver and anti-duck hepatitis virus rabbit serum previously absorbed with normal chicken embryo liver, Murty (1960) reported that two lines of precipitation, supposedly specific for the virus, were produced. Serum from ducks which had recovered from natural infection produced only one line.

Buthala (1956) demonstrated seven lines of precipitation when infectious bronchitis virus (IBV) infected allantoic fluid was diffused against anti-IBV rabbit serum. None of the lines was considered by the author to be specific for the virus. Normal allantoic fluid produced only five lines. After trypsinization or heating IBV for 10 minutes at 100 C, only three lines were produced. Treatment of the virus with ether and potassium periodate did not reduce the number of lines.

MATERIALS AND METHODS

Antigens

The following types of infectious bronchitis virus (IBV) referred to by the Michigan State University repository code numbers, were employed.

Type 41 was originally isolated by Dr. Henry Van Roekel, University of Massachusetts, in 1941, and had been maintained in serial passage in chickens. The third chicken embryo passage, embryo infective dose$_{50}$ (EID_{50}) 10^7 to 10^8, was used.

Type 42 was originally isolated by Dr. F. R. Beaudette, Rutgers University, in 1937, and had been through hundreds of chicken embryo passages but the exact number is unknown. The EID_{50} was from 10^7 to 10^8. The 8th passage of type 42 in chicken embryo kidney cell culture (CEKC) was also used. The CEKC ID_{50} was from 10^8 to 10^9.

Type 46 was isolated from a commercial vaccine containing the Connecticut strain identified as A5968 by Jungheer *et al.*, (1956). The virus was in the 12th egg passage and the EID_{50} was from 10^6 to 10^7.

Controls consisted of normal allantoic fluid (NAF) and cell culture medium (CCM) (0.5% lactalbumin hydrolysate, 2% bovine serum, 50,000 units of penicillin and 0.5 mg streptomycin per 100 ml).

Strain GB of Newcastle disease virus (NDV) and a strain of laryngotracheitis virus (LTV) cultivated in chicken embryos were used as controls and also to determine if there was any antigenic relationship to IBV.

All strains of IBV were propagated in 10-day-old chicken embryos using 0.2 ml of a 10^{-2} dilution of the virus. Dilutions were made in nutrient broth and embryos were inoculated via the allantoic cavity. The infected allantoic fluid was collected from living embryos at the 24th to 26th hour post inoculation for strain 42, and at the 34th to 36th hour for strains 41 and 46. The embryos were chilled at 4 C for at least 4 hours before collecting the allantoic fluids which were then pooled for each strain and stored at -60 C.

Normal allantoic fluid was harvested from embryos of the same age as those infested with the virus and stored at -20 C. The embryos were chilled for at least 4 hours before the fluid was collected.

Newcastle disease virus was cultivated the same as IBV 41 and 46 types. The infected allantoic fluid was harvested from dead embryos after chilling them for 4 hours at 4 C. The fluid was then pooled and stored at -60 C.

Laryngobracheitis virus was cultivated on the chrioallantoic membrane of 10 day old chicken embryos. The membranes were collected from living embryos on the 5th day after inoculation and ground in a Tenbroek grinder using nutrient broth as the diluent. The mixture was centrifuged

at 1430 x G for 15 minutes at 4 C. The supernatant fluid was removed and stored at -60 C.

The 86th cell culture passage of IBV 42 was propagated in CEKC. The cultural medium contained 0.5 per cent lactalbumin hydrolysate with 2 per cent bovine serum and 50,000 units of penicillin and 0.5 mg streptomycin per 100 ml. Extracellular virus was collected at 48 hours and stored at -60 C.

The EID_{50} of IBV and of NDV was determined by inoculating 10 day old chicken embryos with serial 10 fold dilutions of the viruses. Dilutions were made in nutrient broth and five embryos were inoculated per dilutions (Cunningham, 1960a). The positive response for IBV 42 and NDV was embryo mortality. For IBV 41 and 46, the positive responses were embryo mortality, curling of the embryos with a wry neck, deformed feet compressed over the head, thickened amnionic membrane and urates in the kidneys.

All antigens at the time of use were thawed at room temperature and centrifuged at 1400 x G for 15 minutes at 4 C. For IBV, the supernatant fluids were removed and dialyzed against two changes of 0.005 M phosphate buffered saline, pH 7.2, for at least 10 hours. The fluid was further dialyzed against a 20 per cent solution of polyvinylpyrrolidone (PVP) in 0.005 M phosphate buffered saline, pH 7.2, until the fluid was reduced to 1/10 of the original volume. The IBV antigens treated in this manner produced lines of

precipitation of much greater intensity than if the viral infected fluids were not dialyzed. Newcastle disease virus and LTV were not dialyzed as they produced lines of intensity comparable to dialyzed IBV antigens.

Heat treatment. Two ml portions of dialyzed IBV 41, 42, and cell cultured IBV 42 were placed in a water bath at 56 C for 30 and 60 minutes. Immediately after removal from the water bath, the virus was chilled in an ice bath.

Trypsin treatment. One ml of 1 per cent Difco trypsin (1:250) was added to 2 ml each of IBV 41 and 42 which were then incubated in a water bath at 56 C for 30 and 60 minutes. The mixtures were removed from the water bath and 1 ml of 1 per cent egg white trypsin inhibitor (Nutritional Biochemicals, Inc.) was added.

Antisera

Rabbits were used for the production of anti-IBV sera. Serum was collected from the rabbits before inoculation with non-dialyzed or concentrated IBV 41 and 46 infected allantoic fluids. The rabbits were inoculated tri-weekly for 4 weeks. Each week, the first injection was given intramuscularly in the thigh, the second intrapertioneally and the last injection intravenously. For the first, second, third, and fourth week, the inocula were 0.5 ml, 1 ml, 2 ml, and 3 ml, respectively. Ten days later, 5 ml was injected intravenously into

each rabbit. Seven days after the last injection, blood samples were collected by cardiac puncture. The rabbits had been fasted for 24 hours prior to bleeding.

The anti-IBV 42 and anti-NAF sera produced by the above procedure were not found satisfactory as very weak lines of precipitation were produced with them. In an attempt to produce good precipitating antisera, the dialyzed and concentrated IBV 42 and NAF were mixed in equal proportions with Bacto-Adjuvant Complete (Freund). One ml of this mixture was inoculated subcutaneously into several places around the neck of the rabbits. Twenty days later, similar injections were made using one ml of a mixture of equal parts of the antigens and Bacto-Adjuvant Incomplete (Freund) which did contain killed Mycobacterium butyricum. Seven days later, blood was collected. The rabbits were fasted for 24 hours prior to bleeding.

All antisera were centrifuged at 500 x G for 15 minutes., passed through a Seitz filter and stored at -20 C.

Diffusion Medium

The diffusion medium, 0.70 gm of IONAGAR no. 2 (Consolidated Labs, Inc.) in 100 ml of 0.15 M phosphate buffered saline, pH 7.2, was autoclaved at 121 C for 15 minutes. To each 99 ml of the medium, one ml of 1-100 aqueous merthiolate solution was added. The medium was colled to 50 C and the diffusion plates prepared.

Preparation of Diffusion Plates

Diffusion plates were prepared using both 100 x 15 mm disposable sterile plastic Petri dishes (Falcon Plastics Co.) and 3-1/4" x 4" Kodak lantern slides.

Petri dish method. Diffusion medium, 25 ml, was poured into 100 x 15 mm plastic Petri dishes and was allowed to harden. The dishes were put in a humidity chamber at 4 C for at least 4 hours prior to cutting of the wells which were 9 mm in diameter and 1 cm apart. Cutting was accomplished by means of a cork borer. It was not necessary to seal the bottom of the wells with diffusion medium since the reactants did not leak between the plate and the medium.

Glass plate method. Kodak lantern slides, 3-1/4" x 4", were boiled in Tide, rinsed thoroughly in running tap water and then washed in six changes of distilled water. The slides were placed in absolute alcohol over-night and dried with a lintless cloth. Using a 10 ml pipette, 10 ml of diffusion medium was uniformally spread on each slide to a thickness of about 2 mm. The slides were kept in a humidity chamber at 4 C for at least 4 hours before the wells were cut. Paper templates were used as a guide for cutting the wells. The templates usually consisted of a 4 mm wide central trench pattern through the 3" length of the slide, with 7 mm diameter circles on each side at a distance of 1 cm from the trench. The slides were placed on the template and the wells and trench

were cut, respectively, with a cork borer and a Bard-Parker knife. The agar plugs were removed. The bottoms of the wells and trenches were not sealed with the diffusion medium.

Test Procedures

Depending upon the particular test, wells of the diffusion plates were filled with antigen, antisera, or normal serum, using a tuberculin syringe. For the wells of the dishes 0.2 ml was used, but for the slides 0.05 ml was used. In all cases, 0.1 ml of antiserum was placed in the trench of the slides.

After the reactants were placed in the diffusion plates, the plates were put in a humidity chamber at room temperature. After 24 hours, 0.1 ml of the test material was added to the wells of the dishes. The wells and trenches of the slides were refilled three times with 0.05 ml of the antigen and 0.1 ml of the antiserum, respectively, at intervals of two hours. The dishes were incubated for 12 days and slides for 5 days.

The plates were examined using a viewing box which is described under the "Photography" section.

Staining of Precipitation Lines

Preparations on the glass slides were stained to reveal lines which were otherwise not detectable, and also to preserve the specimen. The slides were washed in slightly alkaline physiological saline for 36 hours to remove any

antigen and antiserum which had not precipitated. They were then transferred to a distilled water bath. After 4 hours, the slides were removed and a piece of Whatmann No. 1 wet filter paper was placed on the surface of the agar to absorb the water from the agar. Care was taken to prevent the trapping of air bubbles between the filter paper and the agar. The slides were then allowed to dry at room temperature until all the moisture was removed from the agar. The filter paper was removed from the dried agar surface and the slides were stained in a bath containing 0.2 per cent Ponceau S in 3 per cent aqueous trichloroacetic acid for at least one-half hour. The slides were then washed in a 5 per cent acetic acetic acid bath to destain the background. The destaining solution was changed several times until the background of the slide was clear. The slides were then dried in an incubator at 37 C.

Photography

The lines of precipitation were observed with a viewing box which provided diffused light. A wooden box 10" x 10" x 7" deep was painted black and a 10 cm diameter hole was cut in the center of the top of the box. A circular 9" diameter fluorescent light was fixed 2" below the top of the box. The circular tube provided light of uniform intensity from all sides.

The lines of precipitation in the dishes were photographed on 35 mm Kodak high contrast copy film using a

Leica III G 35 mm camera fitted with a universal focusing bellows and a 90 mm lens. The enlarged prints were made on Kodabromide F-3 single weight photographic paper.

Some of the slides with stained lines of precipitate were photographed by placing them directly in an enlarger. The enlarged projection prints of the lines were made using Kodabromide F-3 single weight photographic paper. Some slides could not be photographed in the above manner since the area covering the lines of precipitation was much greater than the enlarger opening could project. Therefore, contact prints were made by placing the slides directly upon Velox F-4 contact print paper and exposing to light from the enlarger.

RESULTS

When IBV 41 and NAF were diffused against anti-IBV 41 rabbit serum, two lines of precipitation occurred near the well containing the virus but not the well containing NAF indicating that they were formed by the antigens in the viral-infected allantoic fluid only (Fig. 1). The lines were concave towards the virus well.

Precipitation lines were formed by NAF and viral-infected allantoic fluid near the antiserum well (Fig. 1). The lines fused in the center to form a continuous arc on the virus and NAF sides of the well indicating that common antigens were present. The lines started to appear within twenty-four hours. By the 4th day the lines had fused to form a band, and it was not possible to determine the number of lines within the band. The lines near the virus well first appeared on the 3rd or 4th day.

Normal rabbit serum did not produce lines of precipitation between virus or NAF (Fig. 1).

When IBV 42 and NAF were diffused against anti-IBV 42 rabbit serum, two lines of precipitation were produced near the virus well (Fig. 2). The lines were of equal intensity and were concave towards the virus well.

The lines produced by the antigens common to NAF and IBV 42 formed a continuous arc of identity on both sides of

the antiserum well (Fig. 2).

Normal rabbit serum did not produce lines of precipitation between virus or NAF (Fig. 2).

Two precipitation lines of low intensity were produced between IBV 46 and anti-IBV 46 rabbit serum (Fig. 3). No precipitation occurred between the NAF and the antiserum near the NAF well. The broad ring around the antiserum well was probably due to the spread of the serum from the bottom of the well.

Normal rabbit serum did not produce lines of precipitation between virus or NAF (Fig. 3).

Diffusion of IBV 42 and NAF against anti-IBV 42 and anti-NAF sera produced lines of precipitation which formed a continuous arc of identity near the antiserum wells. The precipitation lines of the arc formed by IBV 42 and anti-IBV 42 serum were of a higher intensity probably due to a higher concentration of the reactants (Fig. 4).

The two lines formed between IBV 42 and anti-IBV 42 serum near the virus well, were not present between IBV 42 and anti-NAF serum, thus indicating that these lines were formed by the antigens in the viral-infected allantoic fluid and their specific antibody (Fig. 4).

When NDV, LTV, IBV 42, NAF, and cell culture medium were diffused against anti-IBV 42 rabbit serum, lines of precipitation common to all antigens of chicken embryo origin were produced near the antiserum trench (Fig. 5). The lines

of IBV 42 were of a higher intensity than those produced by the other antigens.

Two additional lines were produced only between IBV 42 and anti-IBV 42 rabbit serum. These lines were considered to be specific for the viral antigens in the IBV infected allantoic fluid as they were not evident with the other antigens (Fig. 5).

No precipitation occurred between cell culture medium and anti-IBV 42 serum.

The above results indicate that there were no antigens common to the cell culture medium, NAF or IBV-infected allantoic fluid (Fig. 5).

It is clear from the above experiments that lines of precipitation due to antigens of chicken embryo origin formed near the antiserum well. Therefore, the description of these lines in the subsequent experiments will be omitted unless otherwise stated.

When IBV 46, 41, and NAF were diffused against anti-IBV 41 rabbit serum, two lines specific to IBV-infected allantoic fluid were produced between IBV 41 and homologous antiserum. Only one line was produced between IBV 46 and anti-IBV 41 serum which was continuous with the proximal line produced by IBV 41 (Fig. 6). The NAF produced very weak lines of precipitation. On the original plate, the lines produced by NAF antigens were identical to the lines formed by the antigens of chicken embryo origin present in IBV-infected allentoic

fluid but some of the detail did not reproduce well in the photograph.

Diffusion of IBV 42 and NAF against anti-IBV 41 serum resulted in the formation of two lines of precipitation, specific to the IBV-infected allantoic fluid near the virus well. The line proximal to the virus well was less intense than the distal line. Both lines were concave towards the well containing virus (Fig. 7).

Diffusion of IBV 41, 42, 46, and NAF against anti-IBV 42 rabbit serum produced two continuous lines of precipitation specific to all the IBV-infected allantoic fluids (Fig. 8). All the lines were concave towards the virus wells.

When cell cultured IBV 42, IBV 42, NAF, and cell culture medium were diffused against anti-IBV 42 rabbit serum, only one line was formed between cell cultured IBV 42 and anti-IBV 42 serum. Two lines were produced with IBV 42. The line produced by cell cultured IBV 42 was continuous with the line formed by IBV 42 distal to the virus well. All lines were concave towards the virus well (Fig. 9).

No lines were formed between cell culture medium and anti-IBV 42 serum (Fig. 9).

Diffusion of IBV 41, 42, 46, and NAF against anti-IBV 46 rabbit serum resulted in the formation of two continuous lines by IBV 46 and 42. Only one line was produced by IBV 41 which was continuous with the proximal line of IBV 42 and 46 (Fig. 10).

No lines of precipitation were produced between cell cultured IBV 42 and anti-IBV 41 and 46 rabbit sera. Photographs were not made of this test.

When cell cultured IBV 42 and IBV 42-infected allantoic fluid heated at 56 C for 30 and 60 minutes and unheated IBV 42-infected allantoic fluid and NAF were diffused against anti-IBV 42 rabbit serum, two lines were formed by IBV 42 heated for 30 and 60 minutes. These lines were continuous with the two lines formed by unheated IBV 42. The single line produced by cell cultured IBV 42 was continuous with the distal line formed by unheated and heated IBV 42 (Fig. 11).

There was no precipitation between IBV 42 treated with 1 per cent trypsin at 56 C for 30 and 60 minutes and anti-IBV 42 rabbit serum. Two lines were formed between untreated virus and antiserum only (Fig. 12).

Two specific lines of precipitation were produced when unheated IBV 41-infected allantoic fluid, IBV 41 heated at 56 C for 30 and 60 minutes, and NAF were diffused against anti-IBV 42 rabbit serum. The line distal to the virus well was of very low intensity and could not be photographed (Fig. 13).

There was no precipitation between IBV 41-infected allantoic fluid treated with 1 per cent trypsin at 56 C for 30 and 60 minutes and anti-IBV rabbit serum (Fig. 13).

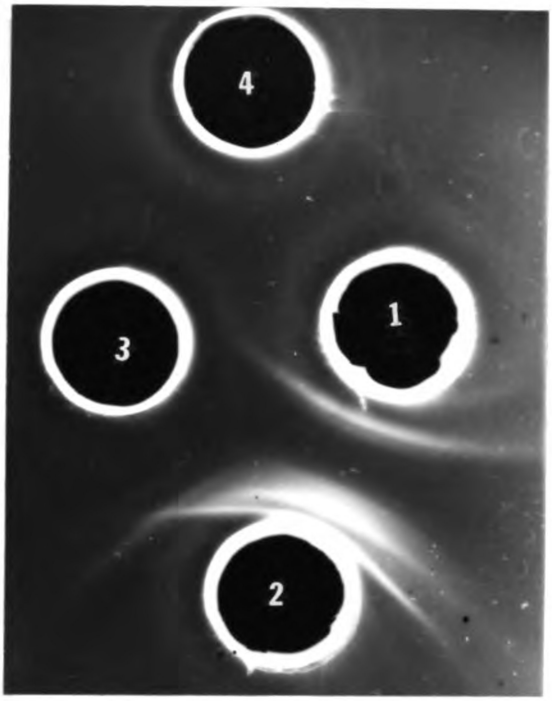

Fig. 1. Diffusion of IBV 41 and NAF against anti-IBV 41 rabbit serum.

1 = IBV 41; 2 = Anti-IBV 41 rabbit serum;
3 = NAF; 4 = Normal rabbit serum.

Fig. 2. Diffusion of IBV 42 and NAF against anti-IBV 42 rabbit serum.

1 = IBV 42; 2 = Anti-IBV 42 rabbit serum;
3 = NAF; 4 = normal rabbit serum.

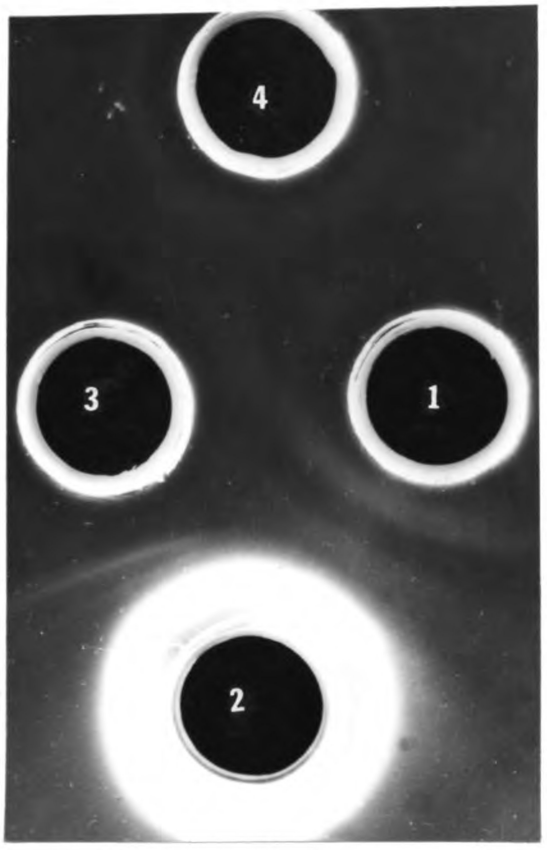

Fig. 3. Diffusion of IBV 46 and NAF against anti-IBV 46 rabbit serum.

1 = IBV 46; 2 = Anti-IBV 46 rabbit serum;
3 = NAF; 4 = Normal rabbit serum.

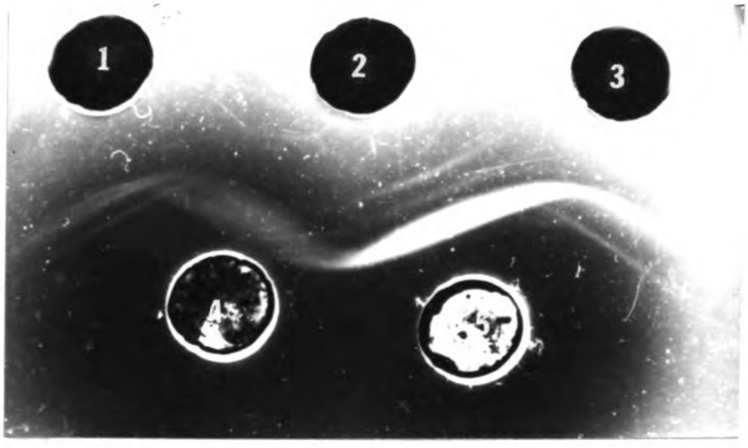

Fig. 4. Diffusion of IBV 42 and NAF against anti_IBV 42 and anti-NAF rabbit serum.

1 and 3 = NAF; 2 = IBV 42; 4 = Anti-NAF rabbit serum; 5 = Anti-IBV 42 rabbit serum.

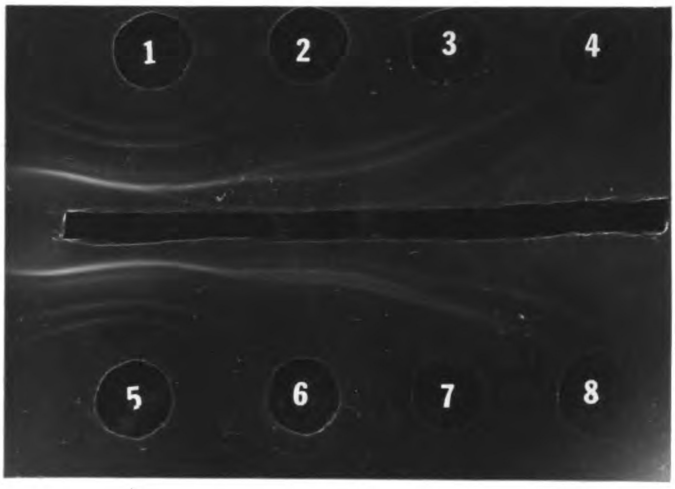

Fig. 5. Diffusion of IBV 42, NDV, LTV, NAF, and cell culture medium against anti-IBV 42 rabbit serum.

1 and 5 = IBV 42; 2 = LTV; 3 and 7 = NAF; 4 and 8 = Cell culture medium; 6 = NDV; 9 = Anti-IBV 42 rabbit serum.

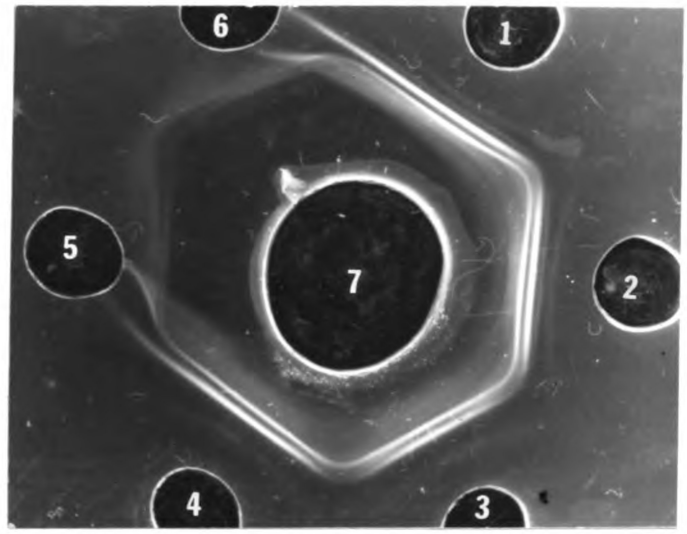

Fig. 6. Diffusion of IBV 46, 41, and NAF against anti-IBV 41 rabbit serum.

1 and 2 = IBV 41; 3 and 4 = IBV 46; 5 and 6 = NAF; 7 = Anti-IBV 41 rabbit serum.

Fig. 7. Diffusion of IBV 42 and NAF against anti-IBV 41 rabbit serum.

1, 3, and 8 = IBV 42; 2, 7, and 9 = NAF;
4, 5, and 6 = Anti-IBV 41 rabbit serum.

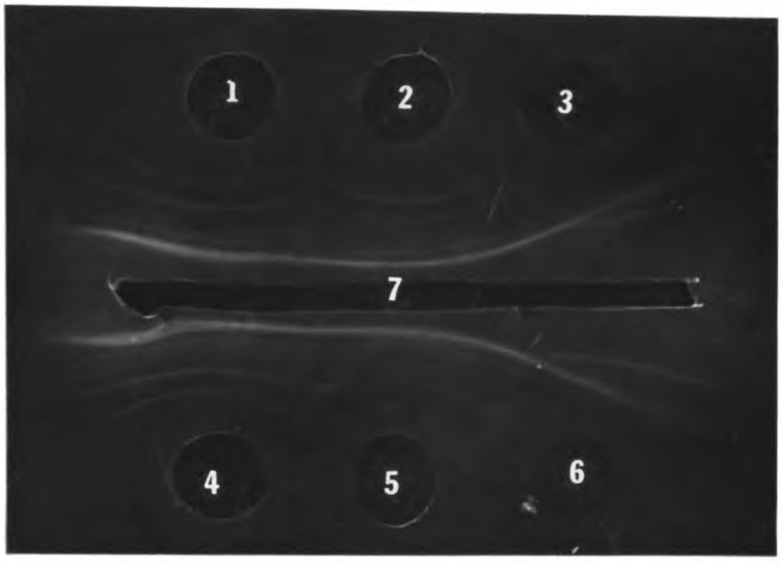

Fig. 8. Diffusion of IBV 41, 46, 42, and NAF against anti-IBV 42 rabbit serum.

1 and 4 = IBV 42; 2 = IBV 41; 3 and 6 = NAF; 5 = IBV 46; 7 = Anti-IBV 42 rabbit serum.

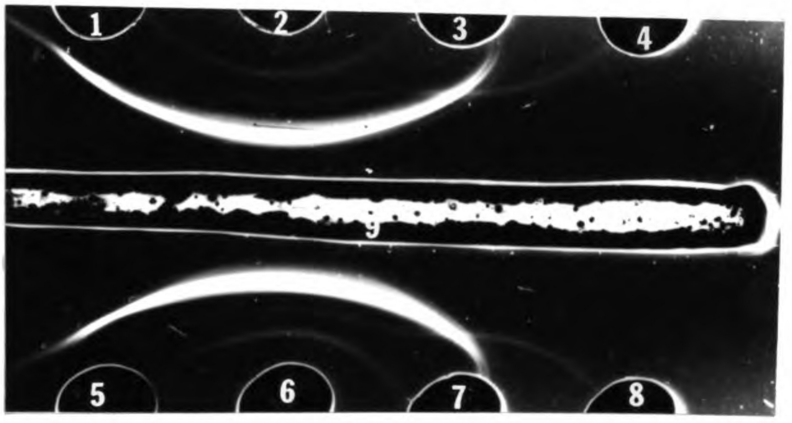

Fig. 9. Diffusion of cell cultured IBV 42, IBV 42, NAF, and cell culture medium against anti-IBV 42 rabbit serum.

1 and 5 = NAF; 2 and 6 = IBV 42; 3 and 7 = cell cultured IBV 42; 4 and 8 = Cell culture medium; 9 = Anti-IBV 42 rabbit serum.

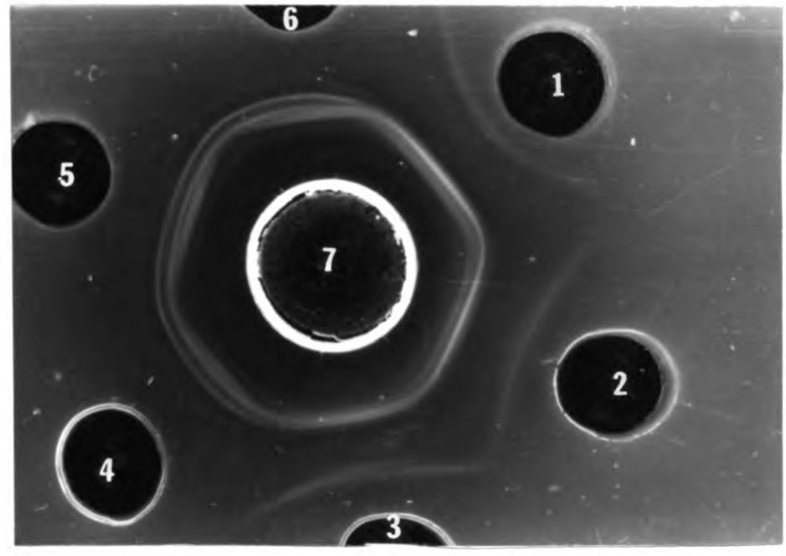

Fig. 10. Diffusion of IBV 41, 42, 46, and NAF against anti-IBV 46 rabbit serum.

1 = IBV 41; 2 = IBV 46; 3 = IBV 42; 4, 5, and 6 = NAF; 7 = Anti-IBV 46 rabbit serum.

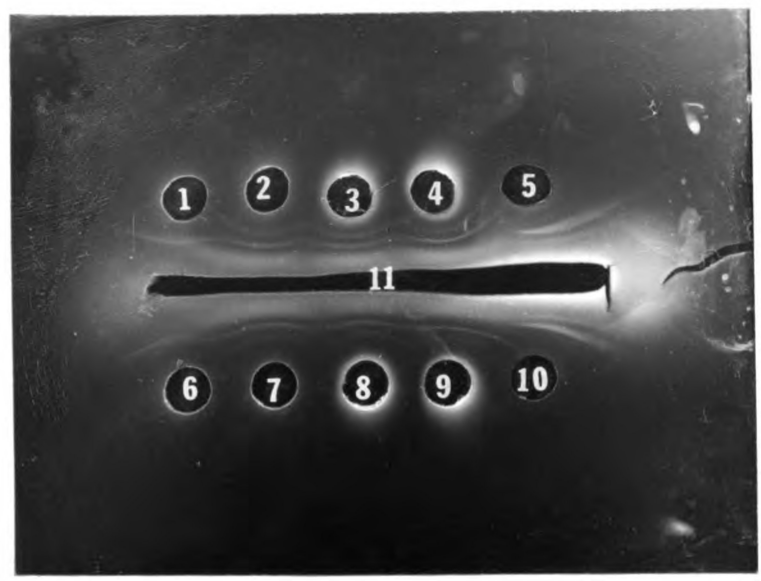

Fig. 11. Diffusion of IBV 42 and cell cultured IBV 42 heated at 56 C for 30 and 60 minutes, unheated IBV 42 and NAF against anti-IBV 42 rabbit serum.

1 = Cell cultured IBV 42 heated at 56 C for 30 minutes; 2 and 7 = IBV 42; 3 and 4 = IBV 42 heated at 56 C for 30 minutes; 5 and 10 = NAF; 6 = Cell cultured IBV 42 heated at 56 C for 60 minutes; 8 and 9 = IBV 42 heated at 56 C for 60 minutes; 11 = Anti-IBV 42 rabbit serum.

Fig. 12. Diffusion of IBV 42, IBV 42 treated with 1% trypsin at 56 C for 30 and 60 minutes, and NAF against anti-IBV 42 rabbit serum.

1 and 5 = IBV 42; 2 and 3 = IBV 42 treated with 1% trypsin at 56 C for 30 minutes; 4 and 8 = NAF; 6 and 7 = IBV 42 treated with 1% trypsin at 56 C for 60 minutes; 9 = Anti-IBV 42 rabbit serum.

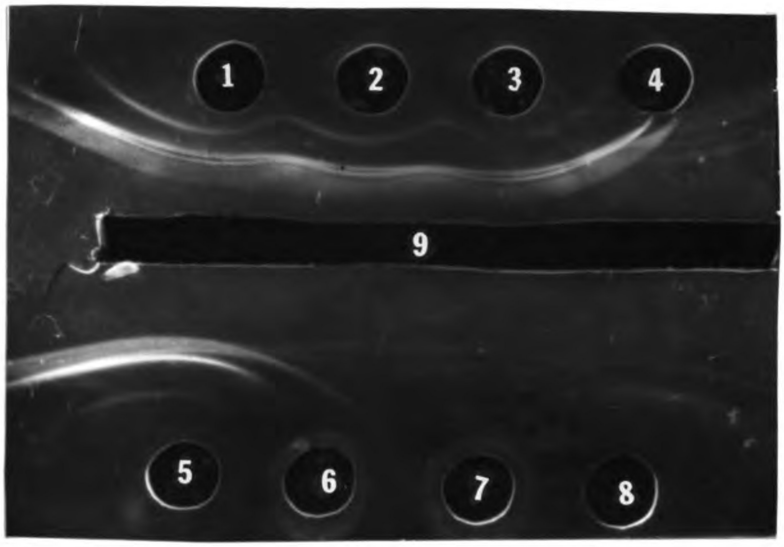

Fig. 13. Diffusion of IBV 41, IBV 41 heated at 56 C for 30 and 60 minutes, IBV 41 treated with 1% trypsin at 56 C for 30 and 60 minutes, and NAF against anti-IBV 41 rabbit serum.

1 and 5 = IBV 41; 2 = IBV 41 heated at 56 C for 30 minutes; 3 = IBV 41 heated at 56 C for 60 minutes; 4 and 8 = NAF; 7 = IBV 41 treated with 1% trypsin at 56 C for 30 minutes; 6 IBV 41 treated with 1% trypsin at 56 C for 60 minutes; 9 = Anti-IBV 41 rabbit serum.

DISCUSSION

The formation of two distinct lines of precipitation specific for IBV 41, 42, and 46-infected allantoic fluid against their homologous antisera indicates the presence of at least two antigens. The number of precipitation lines usually can be interpreted as representing the minimum number of precipitating systems present.

Normal allantoic fluid produced identical lines of precipitation against anti-IBV 42 and anti-NAF rabbit serum. These lines were distinct from the specific lines of precipitation produced by IBV 42 against anti-IBV 42 serum. This indicates that the two antigens specific for IBV-infected allantoic fluid were produced as a result of cultivation of IBV via the allantoic cavity in chicken embryos.

The specificity of the two antigens of IBV in infected allantoic fluid was established by the fact that when NDV-infected allantoic fluid and LTV-infected chorioallantoic membrane homogenate were diffused against anti-IBV 42 rabbit serum, the only precipitation lines produced were those common to the antigens in noraml allantoic fluid. If the antigens were non-viral and had been produced by the cells lining the allantoic cavity as the result of multiplication

of IBV then they should also have been present in the NDV and LTV antigens.

The finding of two antigens specific for IBV is in disagreement with the findings of Buthala (1956), that there are no differences between the number or nature of the antigens in IBV-infected allantoic fluid as compared to normal allantoic fluid when diffused against anti-IBV rabbit serum.

On the basis that the diffusion rate of a substance decreases with increasing molecular weight, the slower moving antigen of IBV, which forms a line of precipitation proximal to the virus well, is probably of a higher molecular weight than the faster moving antigen distal to the virus well.

The time of appearance and the concavity of the lines of precipitation toward the virus well indicate that the molecular weight of the two antigens is higher than that of antibody. This may be explained on the basis of relative diffusion coefficients of the two reactants. If the diffusion coefficient of an antigen is less than that of its antibody, the ends of the arc of the line of precipitation will be away from the lower molecular weight reactant in proportion to the difference between reactant diffusion coefficients.

The faster moving antigen specific for IBV 42-infected allantoic fluid was serologically identical to the single

viral antigen in cell cultured IBV 42 when both were diffused against anti-IBV 42 rabbit serum. This provides further evidence in favor of the viral nature of the antigen specific to IBV 42. No precipitation occurred between cell culture medium and anti-IBV 42 rabbit serum. The presence of only one antigen in cell cultured IBV 42 as compared to two antigens in IBV 42-infected allantoic fluid may be a reflection of some modification of the virus as the result of adaptation to and serial passage in chicken embryo kidney cells.

The two antigens specific for IBV 42-infected allantoic fluid are identical with both the specific antigens of IBV 41 and 46 when all three are diffused against anti-IBV 42 rabbit serum. The two specific antigens of IBV 42 produce two lines of precipitation when IBV 42 is diffused against anti-IBV 41 and 46 rabbit serum. The slow moving antigen of IBV 41 and 46 was identical when both were diffused against anti-IBV 41 and 46 rabbit serum. The fast moving antigen of IBV 41 and 46 is type specific and was not detected when IBV 41 and 46-infected allantoic fluid were diffused against the heterologous antisera. The fact that the fast moving antigens of IBV 41 and 46 were not detected by heterologous antisera indicates that IBV 41 and 46 represent two serotypes. The identity of the fast moving antigen of IBV 41 and 46 to the fast moving antigen of IBV 42 indicates that the latter had been modified during embryo passage.

Lines of precipitation were not produced between cell cultured IBV 42 and anti-IBV 41 and 46 rabbit serum. This may have been due to a low concentration of virus.

Inactivation of IBV 41 and 42-infected allantoic fluid, and IBV 42 in cell culture medium at 56 C for 30 and 60 minutes did not reduce the capacity of the virus to precipitate antibody.

The lack of precipitation of antibody by IBV 41 and 42-infected allantoic fluid treated with 1 per cent trypsin at 56 C for 30 and 60 minutes may be due to removal of the protein coat of the virus as previously reported in electron microscopy studies of IBV (Nazerian, 1960). If this is the case, the protein coat of IBV is involved in the precipitation reaction and not components of the virus underlying this coat.

SUMMARY

1. Two antigens specific for IBV 41, 42, and 46-infected allantoic fluid have been detected using the agar gel diffusion test.
2. Infectious bronchitis virus 41 and 46 in early chicken embryo passage have one common antigen and one unrelated antigen which can be differentiated using reciprocal agar gel diffusion tests with heterologous sera.
3. The identity of IBV 41 and 46 to IBV 42 using anti-IBV 42 serum indicates that the virus is modified during serial passage in chicken embryos.
4. Detection of one antigen in cell cultured IBV 42 by anti-IBV 42 rabbit serum indicates that the virus is modified during serial passage in chicken embryo kidney cells.
5. Antigens specific for IBV 41 and 42-infected allantoic fluid and cell cultured IBV 42 retain the capacity to precipitate with antibody after heating at 56 C for 30 and 60 minutes.
6. Failure of trypsin-modified IBV 41 and 42-infected allantoic fluid to precipitate with antibody is probably due to the removal of the protein coat. If this is true, this indicates that the protein coat and not the underlying components, is involved in the precipitation reaction.

BIBLIOGRAPHY

Aladjem, F. and Lieberman, M. 1952. The antigen-antibody reaction. I. The influence of sodium chloride concentration on the quantitative precipitin reaction. J. Immunol., 69: 117-130.

Bjorklund, B. 1952. Specific inhibition of precipitation as an aid in the antigen analysis with gel diffusion methods. Proc. Soc. Expt. Biol. & Med. 79: 319-324.

Bodon, L. 1955. Typing of foot and mouth disease virus with the agar gel diffusion method. Acta Vet. Acad. Sic. Hung., 5: 157-159.

Boyd, W. C. 1956. "Fundamentals of immunology," 3rd ed. 776 pp. Interscience, New York.

Brown, F. and Crick, J. 1957. Specific reactions with the viruses of foot and mouth disease and vesicular stomatitis. Nature, 179: 316-318.

_____. 1958. Application of agar gel precipitation tests to the study of the virus of food and mouth disease. Virology, 5: 133-144.

Buthala, D. H. 1956. Some properties of the avian bronchitis virus. Ph. D. thesis, Iowa State College, Ames.

Corbo, L. J. and Cunningham, C. H. 1959. Hemagglutination by trypsin-modified infectious bronchitis virus. Am. J. Vet. Res., 20: 876-883.

Crowle, A. J. 1958. A simplified micro double diffusion agar precipitation technique. J. Lab. Clin. Med., 52: 784-878.

_____. 1961. "Immunodiffusion," 1st ed., Academic Press, Inc., New York.

Cunningham, C. H. 1957. Symposium on immunization against infectious bronchitis virus. I. Some basic properties of infectious bronchitis virus. Am. J. Vet. Res., 18: 648-654.

Cunningham, C. H. 1960a. "A laboratory guide in virology," 4th ed. Burgess Publishing Co., Minneapolis.

_____. 1960b. Recent studies on the virus of infectious bronchitis. Am. J. Vet. Res., 21: 498-503.

Elek, S. D. 1948. The recognition of toxogenic bacterial strain in vitro. Brit. Med. J., 1: 493-496.

Ferguson, G. H. 1958. Growth of infectious bronchitis virus in suspended chorioallantoic membranes. M. S. thesis, Michigan State University, East Lansing.

Fahey, J. E. and Crawley, J. F. 1956. Propagation of infectious bronchitis virus in tissue culture. Canad. J. Microbiol., 2: 503-510.

Forsek, M. Z. 1958. Primzena gel difuzionog precipitin tests u dokaz avianjsk kugi. (Demonstration of swine fever virus by gel diffusion precipitin test.) Vet. Glasn., 12: 867. Cited from Vet. Bulletin, 1959, 29: 373.

Gispen, R. 1955. Analysis of pox virus antigens by means of double diffusion. A method for the direct serological differentiation of cow pox. J. Immunol., 74: 134-141.

Graber, P. 1959. Immunoelectrophoretic analysis. Methods of Biochem. Anal., 7: 1-38.

Grasset, E., Pongrats, et Brechbuhler, T. 1596. Analyse immuno-chimique des constituents des venins de serpents par la methode de precipitation eu milieu gelifie. Ann. Inst. Pasteur., 91: 162-186.

Grasset, E., Bonifas, V., and Pongratz, E. 1958. Rapid slide precipitation micro reaction of poliomyelitis and antiserum in agar. Proc. Soc. Exptl. Biol. & Med., 97: 72-77.

Grishenkova, A. S. 1958. Agar precipitation reaction for the laboratory diagnosis of swine fever. Proc. Lein Acad. Agri. Sci., 23: (6) 35. Cited from Vet. Bulletin, 29: 436.

Hofstad, M. D. 1958. Antigenic differences among isolates of infectious bronchitis virus. Am. J. Vet. Res., 19: 740-743.

Jensen, K. E. and Francis, T., Jr. 1953. Antigen antibody precipitates in the solid medium with influenza virus. J. Immunol., 77: 321-325.

Jungherr, E. L., Chomiak, T. W., and Luginbuhl, R. E. 1956. Immunologic differences in strains of infectious bronchitis virus. Proc. 60th Ann. Meet. U. S. Livestock San., A: 203-209.

Le Bouvier, G. L. 1957. Polio virus precipitins. A study by means of diffusion in agar. J. Exptl. Med., 106: 661-675.

Le Bouvier, G. L., Schwerdt, F. L., and Schaffer, F. L. 1957. Specific precipitates in agar with purified polio virus. Virology, 4: 590-593.

Mansi, W. 1958. Slide gel precipitin test. Nature, 181: 1289.

Merchant, I. A. and Packer, R. A. 1961. "Veterinary Bacteriology and Virology," 6th ed., Iowa State University Press, Ames.

Muldoon, R. L. 1960. Some characteristics of hemagglutinating activity of infectious bronchitis virus. Ph. D. thesis, Michigan State University, East Lansing.

Murty, D. K. 1960. Some serological methods of duck virus hepatitis. M. S. thesis, University of Illinois, Urbana.

Nazerian, K. 1960. Electron microscopy studies of the virus of infectious bronchitis and erythrocytes agglutinated by trypsin-modified virus. M. S. thesis, Michigan State University, East Lansing.

Oakley, C. D. and Fulthorpe, A. J. 1953. Antigenic analysis by diffusion. J. Pathol. Bacteriol., 65: 49-60.

Oshel, D. D. 1961. A study of some variables in the neutralization test as used for potency testing commercially available infectious bronchitis vaccines. M. S. thesis, Michigan State University, East Lansing.

Ouchterlony, O. 1948. In vitro method for testing the toxin producing capacity of Diphtheria bacteria. Acta Pathol. Microbiol. Scand., 25: 186-191.

_____. 1958. Diffusion-in-gel methods for immunological analysis. Progr. Allergy, 5: 1-78.

Oudin, J. 1946. Methods d'analyze immunochimique par precipitation specifique en milieu galifie. Compt. rend. Acad. Sci., 222: 115-116.

Oudin, J. 1952. Specific precipitation in gels and its application to immunochemical analysis. Methods Med. Research, 5: 335-378.

Ozawa, Y. 1959. Some properties of infectious bronchitis virus propagated in isolated chorioallantoic membrane. Ph. D. thesis, Michigan State University, East Lansing.

Reagan, R. L., Brueckner, A. L., and Delaplane, J. P. 1950. Morphological observations by electron microscopy of the virus of infectious bronchitis of chickens and chronic respiratory disease of turkeys. Cornell Vet., 40: 384-386.

Reagan, R. L. and Brueckner, A. L. 1952. Electron microscope studies of four strains of infectious bronchitis virus. Am. J. Vet. Res., 13: 417-418.

Randall, J. E. 1958. "Elements of Biophysics," 33 pp. Year Book Publ., Chicago, Illinois.

Singh, I. P. 1960. Some properties of infectious bronchitis virus as determined by thermal and formalin inactivation. Ph. D. thesis, Michigan State University, East Lansing.

Spring, M. P. 1960. Cultivation of infectious bronchitis virus in chicken embryo kidney cells. M. S. Thesis, Michigan State University, East Lansing.

Stone, S. S. 1960. Multiple components of rinderpest virus as determined by the precipitin reaction in agar gel. Virology, 11: 639-640.

Van Rooyan, C. E. 1954. A revision of Holme's classification of animal viruses, sub Order III (Zoophagineae). Canad. J. Microbiol., 1: 227-230.

White, C. 1958. A specific diffusible antigen of rinderpest virus demonstrated by agar double diffusion precipitating reaction. Nature, 181: 1409.

Wittmann, G. 1958. Der hachwis prazipitiender Antikorper bei der Hicheer pocken infection mit hilfe de agar diffusixns ver falrens. (Demonstration of the precipitating antibodies with fowl pox by the agar diffusion technique). Zbl. Vet. Med., 5: 769. Cited from Vet. Bulletin, 1959, 29: 45.