ELECTRON MICROSCOPY STUDIES OF THE VIRUS OF INFECTIOUS BRONCHITIS AND ERYTHROCYTES AGGLUTINATED BY TRYPSIN MODIFIED VIRUS

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By

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A THESIS

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This thesis is

respectfully dedicated

to

MY FAMILY

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TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Infectious bronchitis virus (IBV) does not agglutinate erythrocytes, but modification of the virus by trypsin will induce the hemagglutinating activity (HA) of the virus which is inhibited by normal and specific immune serum.

The objectives of the study were: (1) to determine the morphological characteristics of the virus before and after modification by trypsin, and (2) to study the morphological feature of the virus-erythrocyte interaction.

LITERATURE REVIEW

Infectious Bronchitis

Infectious bronchitis is a respiratory disease of chickens caused by the virus Tarpeia pulli (von Rooyen, 1954). Infectious bronchitis virus (IBV) can be readily propagated in embryonating chicken eggs (Beaudette and Hudson, 1937; Cunningham and Stuart, 1947; Cunningham and El Dardiry, 1948) and cell culture (Fahey and Crawley, 1956; Cunningham, 1957, 1960). When first isolated in embryos by several routes of inoculation, the virus produces gross alterations such as curling and stunting of the embryo, fibrosis of the amnion, improper development of the feathers, and urates in the kidney and mesonephros (Beaudette and Hudson, 1937; Delaplane and Stuart, 1939; Fabricant, 1949; Loomis et al., 1950). Mortality of embryos is variable. With serial passage in embryos the virus increases in pathogenicity for the embryo and mortality is the characteristic finding (Beaudette and Hudson, 1937; Delaplane and Stuart, 1939). Completely egg-adapted virus kills all embryos but is not immunogenic. It is neutralized by specific immune serum.

The virus can also be propagated in the isolated chorioallantoic membrane (CAM) (Ferguson, 1958; Ozawa,

1959), monkey kidney cells, and chicken embryo kidney cells (CEKC) (Fahey and Crawley, 1956; Spring, 1960) but not in mouse and rat liver cells. With the CAM and CEKC, several passages of IBV are necessary for adaptation of the virus to the cultural medium. With CEKC but not the CAM, cytopathic effects are produced by the virus and can be used for infectivity assay (Chomiak et al., 1958; Spring, 1960).

With both embryo and cell culture, there are definite lag, log, stationary and decline phases during multiplication of the virus (Hitchner and White, 1955; Fahey and Crawley, 1956; Spring, 1960).

There are at least two serotypes of IBV, and possibly a third, based on immunogenic studies (Hofstad, 1958). The virus at 4 C is more stabile in an acid medium (pH 3.0 for 14 days) than in an alkaline medium (pH 10.6 for two days) with maximum stability at pH 7.8 for 170 days (Cunningham and Stuart, 1946). Thermal inactivation of IBV in early egg passages follows a bimodal reaction. In the early egg passage the virus exists in O phase, in which particles retain their original identity, and D phase particles which are derived as a result of embryo passage. The O phase is thermostabile while the D phase is thermolabile (Singh, 1960). Horse serum has a protective effect on the virus against heat (Hofstad, 1956).

The virus in allantoic fluid is from 60 to 100 mμ in diameter with small projections being most prominent in samples prepared in saline (Reagan and Bruchner, 1952; Reagan et al., 1948). In the CAM in embryo culture, elementary bodies as large as 200 mμ in diameter are present (Domermuth, 1954). The virus may be partially purified and concentrated by trypsinization and ultracentrifugation following "salting out" of the infected allantoic fluid with ammonium sulfate (Buthala, 1956), and adsorption to and elution from anion exchange resin (Mallmann, 1960). The density of IBV is approximately 1.15 (Buthala, 1956). The isoelectric point is at approximately pH 4.15 (Cunningham and Stuart, 1947a; Buthala, 1956).

Hemagglutination

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

Hemagglutination by these viruses is inhibited by specific antiserum, nonspecific factors in normal serum (Chu inhibitor) and a variety of mucoid substances of

biological origin such as human urine, egg-white, allantoic fluid, and the receptor destroying enzyme (RDE) of Vibrio cholera.

Not all viruses possess the ability to agglutinate erythrocytes and there are differences in the mode of action of the viruses which possess this activity. Based on their mode of action on erythrocytes, viruses can be classified into two main groups: (1) those which directly agglutinate cells, and (2) those which indirectly cause agglutination of the cells. The first group is divided into three subdivisions: (a) myxoviruses: influenza, mumps, and Newcastle disease in which adsorption of the virus to the cell and enzymatic action of the virus resulting in receptor destruction, and finally elution of the virus from the cell, are the three successive steps involved in the virus-erythrocyte interaction; (b) some viruses such as those of vaccinia, variola, ectrommelia, and meningopneumonitis in which the hemagglutinin is separable from the virus by ultracentrifugation and ultrafiltration (Lannett, 1959; Anderson, 1959), and is in the form of a soluble lipoid antigen; and (c) some of the arthropod-borne viruses such as Japanese B encephalitis in which the virus particle is believed to be the hemagglutinin. No enzyme is present and there is no receptor destruction or elution of the virus (Anderson, 1959).

Hemagglutination of the second group can be demonstrated only by an indirect method (Lennett, 1959). Herpes simplex and adenoviruses, for example, do not possess direct hemagglutination activity. If sheep erythrocytes are treated with tannic acid and the virus is adsorbed to them, the addition of specific antiserum will cause the cells to agglutinate (Scott et al., 1957; Friedman and Bennett, 1957). This is the opposite of the action of the first group where hemagglutination is inhibited by specific antiserum.

1. Adsorption: The initial rate of adsorption is fast over a range from 4 to 37 C (Buzzell and Hanig, 1958). Physical evidence suggests that adsorption is ionic in nature while chemical observations indicate that attachment is through the hydroxil group of a polysaccharide chain present on the substrate. The pH and salt dependency of adsorption indicates that ionization is responsible for the initial attachment (Burnet and Edney, 1952; Sagik and Levine, 1957). Adsorption can occur only to a limited extent in the absence of salt. Bivalant cations are more effective than monovalant cations (Burnet and Edney, 1952). The firmness of attachment of the virus to the cell is minimum with low concentration of salt at 4 C, but maximum with high concentration of salt (Buzzell and Hanig, 1958). Edney (1949) found that the action of RDE is

reduced at pH 6.5 when a calcium deionizing agent is present. Porterfield (1952) concluded that a trace of calcium is always necessary but is not a specific requirement.

2. Enzymatic action: Hirst (1942) reported that the reaction of influenza virus and erythrocyte is that of an enzyme-substrate complex. Cells once treated with a given virus will not react with the same virus again, but will react with certain other viruses. Hirst compared this to the reaction of an enzyme combining with a substrate, subsequent chemical change in the substrate, and release of the enzyme. Burnet (1952) showed that the loss of agglutinability of erythrocytes by one type of virus is specific to the virus and might not be the same as with another type of virus. A "receptor gradient" was constructed in which the lower member of the gradient could not reagglutinate cells treated with higher members, but the higher members were able to reagglutinate cells treated with the lower members.

Hanig (1948) observed that the electrokinetic mobility of negatively charged human erythrocytes was reduced after treatment with strain PR8 of influenza A virus. A parabolic curve with a high virus-cell ratio, and a sigmoid curve with a low ratio, accompanied the decrease of electrokinetic mobility of the cell. After reaction with one site on the cell, the virus immediately

is adsorbed to another site with a resulting reduction of the electrokinetic potential of the cell. This reduction in the charge of the cell may be the result of the loss of charge, or adsorption of an opposite charge.

3. Elution: With the myxoviruses, receptor destruction is followed by elution of the virus from the cell. Cells thus treated are considered to be stabilized cells and are refractory to the same virus (Hirst, 1942), but will be agglutinated by higher members in the receptor gradient. Elution is slow at 0 C (Stone, 1949), but rapid at 37 C (Hirst, 1942). Cations are necessary for elution (Burnet and Edney, 1952). Complete elution of Newcastle disease virus does not occur at high virus-erythrocyte ratios, thus enabling cells so treated to agglutinate fresh erythrocytes and to be agglutinated by the addition of specific serum (Burnet and Anderson, 1946; Anderson, 1947). It is assumed that elution of the virus is possible only when new receptor sites are available. Anderson (1959) concluded that the surface of the cells so treated may obtain the same functional groups as those of the viral membrane.

Of the three properties of the virus, infectivity, hemagglutination, and elution, infectivity is the least stabile against all inactivating agents. The properties of hemagglutination and elution are usually lost simultaneously, but it is possible to inactivate thermally the

ability of the virus to elute without the destruction of the hemagglutinin (Buzzell and Hanig, 1958). Virus thus treated is termed indicator virus. Lauffer and Carnelly (1946) observed that two hemagglutination components are present in strain PR8 of influenza A virus and that both are inactivated according to a three halves order kinetics. Woese (1956) observed that both components are inactivated according to first order kinetics. Formaldehyde inactivation of PR8 hemagglutinin was studied by Brandon (1957) as a function of pH, temperature, and formaldehyde concentration. The reaction appeared to be complex and approximated first order kinetics.

Infectious bronchitis virus does not possess hemagglutinating activity, but the virus can be modified with trypsin to induce hemagglutination (Corbo and Cunningham, 1959; Muldoon, 1960). According to Corbo and Cunningham (1959), the hemagglutinin is probably present in two fractions: one associated with the virus particle, and the other consisting of the greater part, distinct from the virus particle. The rate of adsorption of the hemagglutinin varies directly with temperature. Elution varies indirectly with temperature. Inhibitors of hemagglutination are present in specific antiserum, normal serum, and in IBV-infected and normal allantoic fluid. Infectivity of the virus for the chicken embryo is reduced almost 98

per cent after trypsin modification. The hemagglutinin of IBV reacts only with chicken and turkey erythrocytes (Muldoon, 1960). Trypsin modified virus is stabile at 56 C for three weeks and retains its ability to agglutinate erythrocytes after six and one-half hours at 56 C prior to modification by trypsin. Infectivity is lost after two hours at 56 C, but the virus is still antigenic for chickens when inoculated intravenously. The highest titer of the hemagglutinin is obtained 72 hours after inoculation of the virus into the allantoic cavity of the chicken embryo, while the infectivity titer is at its maximum 24 hours after inoculation. The hemagglutinin but not the infective particle, is retained by Millipore filters having a pore diameter of 50 mμ. Hemadsorption does not occur. There is no way as yet to destroy the nonspecific inhibitor of the hemagglutinin present in the normal serum to permit assay of antibody by means of the hemagglutination inhibition test.

Electron Microscopy

Electron microscopy has been used not only for studies of the size and shape, but also for studies of the internal structure of viruses, and their relation to the host cell.

Erythrocytes agglutinated by influenza virus

and other viruses of the myxovirus group have been studied by this method in order to demonstrate the morphological features of the virus-erythrocyte interaction as well as studies of the virus and the hemagglutinin particle.

Owing to the thickness of the intact erythrocyte, it is necessary to hemolyze the cell before treatment with the virus, to visualize better the virus-cell interaction. Treatment with saponin or washing the cells in distilled water will remove hemoglobin from the cell. With cells thus treated, the membrane is thin enough to permit the virus adsorbed to the membrane to be readily observed with the electron microscope and to show the virus particles to be arranged at random over the cell surface.

Dowson and Elford (1949) found that the use of hemolyzed erythrocytes treated with influenza virus was a good procedure for studying the morphological characteristics of the virus without the necessity of purification of the virus. The number of particles per unit area of erythrocyte membrane was determined by direct particle count. A saturation level was found for each strain. The number of particles per unit area was proportional to the concentration of the virus and the number of cells, and was a function of both temperature and time of contact. The saturation number has been reported to be as small

as 300 (Hanig, 1948) and as large as 7000 (Bateman et al., 1955).

Donald and Isaacs (1954) found that the filamentous forms of influenza virus also adsorb to the erythrocyte membrane. Filaments ruptured by ultrasonic vibration yielded a greater number of spherical particles and a greater HA titer.

The direct particle count of viruses adsorbed to the cell membrane has been applied in estimating the number of virus particles in the preparation. Using NDV, mumps, fowl plague, and influenza C, Isaacs and Donald (1955) obtained results by direct particle count similar to those obtained by other methods of viral assay. Donald et al. (1954) found that the ratio of virus to cell at the end point of hemagglutination was 1:1. It was concluded that 10 HA units equal one embryo lethal dose.

Tyrrell and Valentine (1957) compared the direct particle count method to the indirect method of Levine et al. (1953) in which the absolute assay was by spectrophotometric measurements, and concluded that the indirect method underestimates the direct particle count by a factor of about 10.

Incomplete forms of influenza virus produced by different methods (Schkesinger, 1950; von Magnus, 1951; Henle et al., 1955) were found to possess the same HA

activity as the standard virus but lacked infectivity. Incomplete virus is less uniform in size and shape than the corresponding standard virus. The mean diameter is, however, of a similar magnitude (Paucker et al., 1959). Incomplete virus may contain only one-third of the quantity of ribonucleic acid carried by the infectious agent (Ada and Perry, 1955), all of which can be accounted for in the soluble antigen (Hoyle et al., 1954).

Hoyle (1950 and 1952) showed that treatment of influenza virus with ether will break the virus into two fractions, both of which are in the aqueous phase. These two fractions are the hemagglutinin and soluble antigen (S antigen). The size of both components was found to be about 12 mμ.

Paucker et al. (1959) treated both infectious and noninfectious forms of strain PR8 of influenza A virus with ether. The virus prior to ether treatment was about 118 mμ. When the infectious particles were treated with ether, two fractions were obtained, the hemagglutinin and the S antigen. The two fractions were completely separated following three successive alternative procedures of adsorption and elution of the hemagglutinin to erythrocytes and concentration by ultracentrifugation. The hemagglutinin consisted of small particles from 35 to 40 mμ, and a few ghost-like particles. This heterogeneity of the

hemagglutinin was also observed from sedimentation studies. The soluble antigen consisted of small, 17 mμ rod-like structures without any subunits. When noninfectious particles were treated with ether, two fractions were obtained as in the case of infectious particles. When the two fractions were separated by adsorption of the hemagglutinin to the erythrocyte, the hemagglutinin consisted mostly of ghost-like structures with a few typical, spherical HA particles. The appearance of the S antigen, however, was not different from that of the complete virus, but it was present in a lesser number than the infective particles. The main difference between the complete and incomplete virus was, therefore, the greater amount of S antigen and typical HA particles in the complete form of the virus. It was concluded that the rod-like structure of the S antigen is not incompatible with the thread forms of nucleic acid, and also that several HA units are present in one infectious particle.

Schafer (1956) treated fowl plague virus with ether and separated the two fractions obtained by adsorption of the hemagglutinin to erythrocytes. The virus prior to ether treatment was about 72 mμ in diameter with three to four per cent ribonucleic acid (RNA). The hemagglutinin obtained from ether treatment of the virus was about 33 mμ with no RNA. The other component, G antigen

or "bound" antigen, was from 10 to 15 mμ and contained 10 to 15 per cent RNA. The S antigen, related to the incomplete form of the virus, had a diameter of 10 to 15 mμ and contained six to 14 per cent RNA.

MATERIALS AND METHODS

Virus

Infectious bronchitis virus strains 40-24 and 41-11 from the North Central Repository at Michigan State University were used because of the previous studies of these strains for induction of hemagglutination by trypsin (Corbo and Cunningham, 1959; Muldoon, 1960). Each strain is designated with two numbers: the first is the Code number of the virus and the second is the number of egg passages following initial isolation from infected chickens. Strain 40-24 was originally isolated at the Department of Microbiology and Public Health, Michigan State University, in 1956. Strain 41-11 was originally isolated at the Department of Veterinary Science, University of Massachusetts, in 1941.

Propagation of Virus

Embryonating chicken eggs nine to eleven days old were used for propagation of the virus using 0.1 ml of undiluted virus inoculum per embryo via the allantoic cavity. The eggs were candled every 12 hours. At 36 hours post inoculation, the eggs containing living embryos

were chilled overnight at 4 C and allantoic fluid was harvested and stored at -30 C until used.

Erythrocytes

Erythrocytes were obtained by cardiac puncture from Single Comb White Leghorns, using two ml of a two per cent solution of sodium citrate as an anticoagulent for each 10 ml of blood. The cells were washed by centrifugation with three changes of 0.85 per cent NaCl and were finally removed from suspension by centrifugation for 15 minutes at 1500 rpm. The supernatant fluid was removed and the packed cells were stored at 4 C as long as three days before being used.

Reagents

1. A one per cent suspension of trypsin (Difco 1:250) was prepared in double distilled water, incubated at room temperature for 15 minutes, passed through a Seitz filter, and stored at -30 C.

2. A one per cent suspension of egg-white trypsin inhibitor (Nutritional Biochemicals, Inc.) was prepared in sterile, double distilled water and stored at -30 C. The suspension was not filtered because of the decrease of activity from filtration.

Diluents

1. Difco Nutrient broth was used for preparing virus dilutions for infectivity assay.

2. Bacto hemagglutination buffer at pH 7.3 was used as the diluent in all HA tests.

Infectivity Assay

Serial ten-fold dilutions of the virus were prepared and five eggs were inoculated per dilution using 0.1 ml per egg via the allantoic cavity. The eggs were incubated at 99 to 99.5 F for five days. Mortality during the first 12 hours post inoculation was considered to be due to nonspecific causes and was not used in calculations of infectivity. The criteria for calculation of the infectivity titer by Reed and Muench method (1938) were mortality, curling and stunting of the embryo, and urates in the kidney.

Hemagglutination Test

Undiluted virus, two ml, was added to one ml of a one per cent suspension of trypsin and the mixture was incubated at 56 C for 30 minutes. Egg-white trypsin inhibitor, one ml of a one per cent suspension, was added to the mixture to stop the action of trypsin. Serial

two-fold dilutions of the modified virus were prepared in 0.85 per cent NaCl. In each of 12 x 75 mm tubes, 0.25 ml of Bacto hemagglutination buffer and 0.25 ml of the corresponding virus dilution were mixed, and 0.5 ml of a 0.2 per cent suspension of erythrocytes in saline was added to the mixture. The tubes were shaken well, incubated at room temperature for 50 minutes, and the reaction was recorded according to the pattern method. The HA titer was the reciprocal of the highest dilution of the virus which showed complete hemagglutination.

Preparation of Specimens for Electron Microscopy

Number 200 EFFA grids were used to support a thin film of Formvar in ethylene chloride as the substrate. After the film was established on the grid and completely dried, it was kept at 4 C until used for preparation of specimens for electron microscopy.

To prepare the specimens of the virus, the suspension was sprayed directly on the film on the grid using a No. 166 Pyrex Nebulizer. For preparation of specimens in which cells were involved, a drop of the cell suspension was placed on the film with a micropipette and the excess liquid was adsorbed with lens paper, leaving the cells attached to the collodion film. The specimens were then dried at room temperature and stored at 4 C for as

long as one day before examination with the electron microscope. Most specimens were shadowcast with tungsten at 30°. An RCA Model EMO electron microscope was used.

RESULTS

Morphological Studies of the Virus Particle

Frozen, viral-infected allantoic fluid was thawed at room temperature and centrifuged for 15 minutes at 2500 rpm. The supernatant fluid was removed and centrifuged for one hour, at 21500 g, in a Model PR-1 International Equipment Co. centrifuge with a multispeed attachment, and a six place rotor containing five ml per tube. The supernatant fluid was removed and collected in a 400 ml prescription bottle. Approximately 320 ml of partially purified virus was prepared. The virus was used for ultracentrifugation using the Specialized Instruments Corporation ultracentrifuge, Model E. The following procedure was used for nine cycles:

Cycle	No. of tubes	Volume per tube	Speed (rpm)	Time	RCF
1	10	35 ml	42040	One hour	114610 g
2	2	35 ml	42040	" "	114610 g
3	1	35 ml	42040	" "	114610 g
4	2	6 ml	42040	" "	114610 g
5	1	6 ml	47660	" "	142210 g
6	1	6 ml	47660	" "	142210 g
7	1	6 ml	47660	" "	142210 g
8	1	6 ml	47660	" "	142210 g
9	1	6 ml	56100	" "	197040 g

After each cycle, about three-fourths of the supernatant

fluid was removed and the sedimented virus was resuspended in sterile, double distilled water. A bluish, opalescent pellet was obtained from the ninth cycle marking the end of ultracentrifugation.

Serial ten-fold dilution of this concentrated virus was made in sterile, double distilled water in order to prevent aggregation of the virus. Electron microscopy of the concentrated virus did not show any appreciable number of virus particles. Only a few aggregates of small particles were observed. Tests for infectivity and hemagglutinating activity of the virus were negative. This indicated that the infectivity and HA activity of the virus were somehow adversely affected during the course of ultracentrifugation as well as the identity of the virus particle as determined by electron microscopy.

Following the negative results obtained with virus as processed above, another sample was partially purified by centrifugation at 21500 g for one hour. The supernatant fluid was removed and serial ten-fold dilutions of the virus were made in sterile, double distilled water. Normal allantoic fluid was subjected to the same procedure.

Electron microscopy of specimens shadowcast with tungsten showed that a 10^{-3} dilution of the virus was the optimum to obtain clear pictures of individual

virus particles. This dilution was used for all subsequent samples.

In the IBV 40-24 infected allantoic fluid, spherical particles 60 to 80 mμ and 20 to 40 mμ were observed (Figure 1). In the normal allantoic fluid spheres 20 and 40 mu were observed (Figure 2). This would indicate that the 60 to 80 mμ particles in the infected allantoic fluid were the virus particles.

Infectious bronchitis virus adsorbs to anion exchange resin in the chloride form and subsequently elutes from the resin in 10 per cent Na_2HPO_4 solution. The eluted virus has an infectivity titer of 0.5 to 1.0 log unit higher than the original virus (Mallmann, 1960). Ten ml of infected fluid and five ml of resin (Dowex, 12K) were shaken intermittently by hand for 15 minutes in a centrifuge tube and then centrifuged at 2500 rpm for 15 minutes. The supernatant fluid was removed and the sediment was restored to original volume with 10 per cent Na_2HPO_4 solution. The mixture was again shaken intermittently for 15 minutes, recentrifuged, and the supernatant fluid removed with a capillary pipette (Mallmann, 1960) for tests of infectivity, HA, and for electron microscopy. With strains 40-24 and 41-11, small and large particles in aggregate form were observed prior to adsorption to and elution from the resin (Figures 1 and 3).

After adsorption to and elution from the resin, only the large particles were present with a few aggregates (Figure 4). There seemed to be more individual virus particles than in the original sample, but this could not be substantiated due to the lack of quantitative procedures. There were, however, fewer aggregates than in the original sample. Comparison of the normal allantoic fluid and IBV infected allantoic fluid before and after elution from the resin showed that only the large particles were adsorbed to the resin. If the small particles were also adsorbed, the attachment was firm and not reversible with Na_2HPO_4. The size and shape of particles after elution confirms that the virus is from 60 to 80 mμ.

When both strains were modified by trypsin before and after elution from the resin, the modified virus was able to agglutinate erythrocytes. During treatment with trypsin, the virus remained spherical but was reduced to 25 to 35 mμ after 30 minutes at 56 C (Figure 5). As a general finding, clear electron micrographs were not obtained from the samples after 5, 10, and 20 minutes because of obscuring of the virus with trypsin and egg-white trypsin inhibitor. It is possible that either a protein coat of the virus was removed through the trypsin digestion and that the free virus particle showed HA activity, or that a mucoprotein inhibitor present on the surface of the virus was removed by trypsin.

The result of infectivity and HA tests with both strains showed a slight decrease in infectivity of the eluted virus but the HA titer remained the same (Tables 1 and 2). From the shape and size of the virus after elution, and the fact that after elution there is no decrease in HA titer, it may be concluded that (1) the hemagglutinin of IBV is associated with the virus particle and is not present in solution as a free antigen separate from the virus particle, and (2) the homogeneity of the virus after elution from resin suggests that there is resemblance of shape and size between infective particles and HA particles even if they are actually independent of each other.

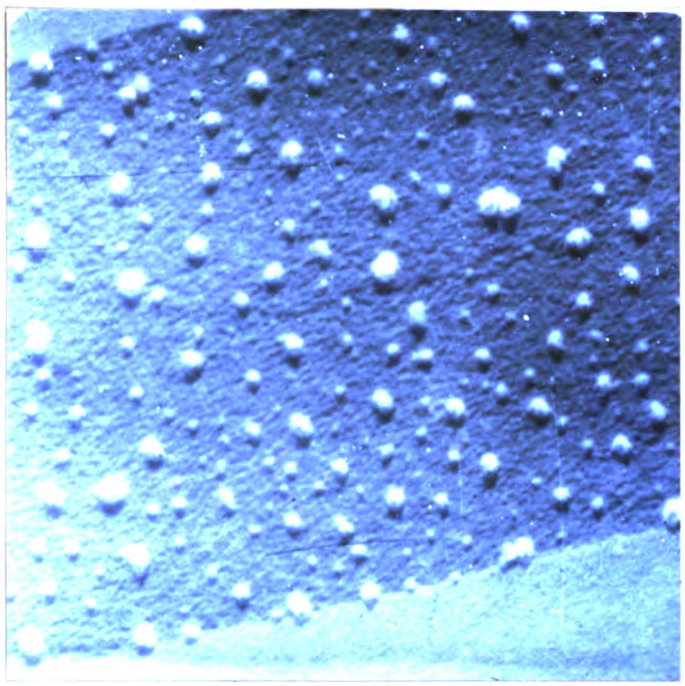

FIGURE 1. Electron micrograph of IBV 40-24 infected allantoic fluid, shadowcast at 30°. X 40000

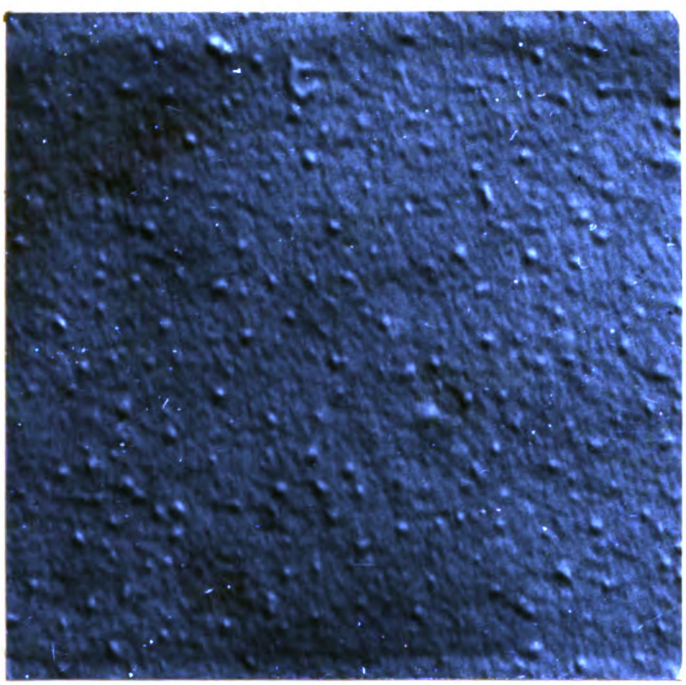

FIGURE 2. Electron micrograph of normal allantoic fluid, shadowcast at 30°. X 40000

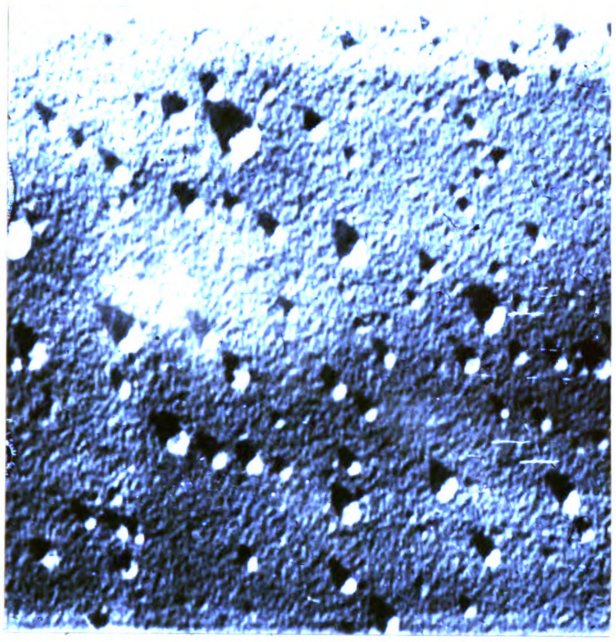

FIGURE 3. Electron micrograph of IBV 41-11 infected allantoic fluid, shadowcast at 30°. X 40000

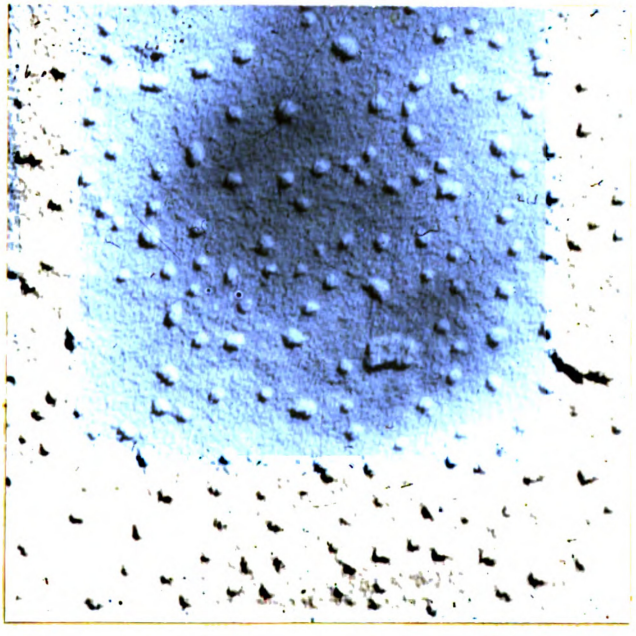

FIGURE 4. Electron micrograph of IBV 41-11 after elution from resin, shadowcast at 30°. X 40000

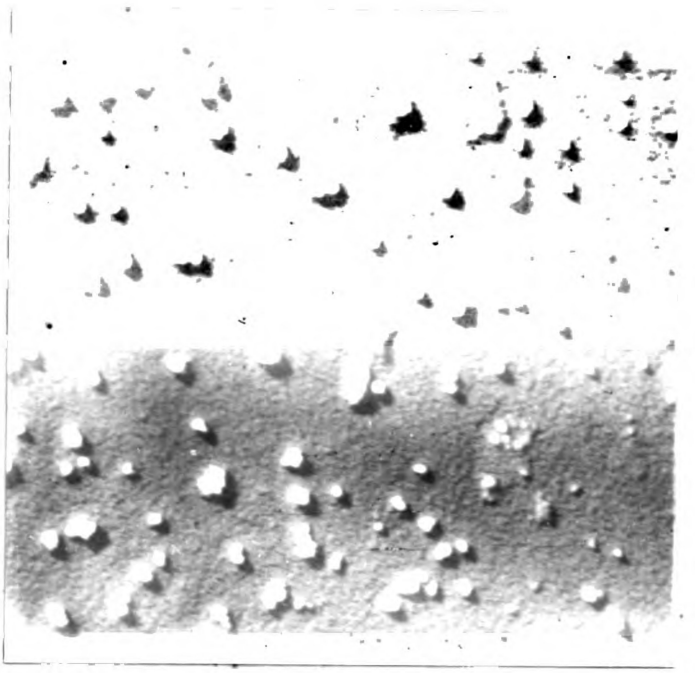

FIGURE 5. Electron micrograph of trypsin-modified IBV 40-24 (30 minutes at 56 C) shadowcast at 30°. X 40000

TABLE 1. Infectivity and HA titers of IBV 40-24 before and after treatment with anion exchange resin.

	Size of the particles	Size of the trypsin modified particles	Infectivity titer	HA titer
Infected allantoic fluid prior to treatment with resin	20 to 80 mμ	25 to 35 mμ	7.2	640
Infected allantoic fluid after treatment with resin	60 to 80 mμ	25 to 35 mμ	6.6	640
Normal allantoic fluid	20 to 40 mμ			

TABLE 2. Infectivity and HA titers of IBV 41-11 before and after treatment with anion exchange resin.

	Size of the particles	Size of the trypsin modified particles	Infectivity titer	HA titer
Infected allantoic fluid prior to treatment with resin	25 to 80 mμ	25 to 35 mμ	6.5	1280
Infected allantoic fluid after treatment with resin	60 to 80 mμ	25 to 35 mμ	6.2	1280

Morphological Changes of Erythrocytes Treated with Trypsin Modified IBV

Morphological studies of erythrocytes agglutinated with the virus did not permit visualization of the virus-cell interaction due to the thickness of the intact erythrocyte. It was, therefore, necessary to hemolyze the cells before treatment with virus. Treatment with saponin or washing the cells in distilled water will remove the hemoglobin from the cell (Dounce et al., 1943). In the present experiment 0.5 per cent NaCl solution was used. After separation of the cells from the serum by centrifugation, they were washed with several changes of 0.5 per cent NaCl and the hemoglobin was gradually released.

Normal and hemolyzed erythrocytes were mixed with trypsin-modified IBV, and incubated at room temperature for 20, 40 and 60 minutes. After each interval, the specimens were examined with the electron microscope following the procedure previously mentioned. In one experiment, the suspension of the cells was allowed to dry on the collodion mount in an attempt to obtain an HA pattern.

1. Normal erythrocytes

a) Not treated: The edges of the cell were smooth without any visible projections. Due to the opacity

of the cell, no internal structure could be observed.

b) Agglutinated cells after reaction with trypsin-modified IBV 40-24 for 20 minutes: These cells were also opaque and nothing could be observed on the horizontal plane, but adsorption of the virus was evident at the periphery of the cell (Figure 8). At this stage, virus was adsorbed to the cell but morphological changes of the cell could not be observed.

c) Agglutinated cells after reaction with trypsin-modified IBV 40-24 for 60 minutes: These cells were also opaque, but a few projections were present from the periphery of the cell, and were intermediate between the cells (Figure 6). These projections were probably produced in different directions but they were not observed because of the opacity of the cell (Figures 7 and 9). Virus had been released from the cell at this stage but was present close to the surface of the cell. It appears that during the first 20 minutes of exposure of the cell to the modified virus, the virus attaches to the cell membrane without morphological changes of the cell being observed. After a longer period of contact, the virus is released from the cell and few projections are produced on the surface of the cell. These projections seem to act as a lattice for attachment of other cells to cause agglutination.

2. Hemolyzed erythrocytes

a) Not treated: A very thin membrane was observed with a fine network on the surface of the cell giving the appearance of small pores to the surface of the cell (Figure 10). An ovoid nucleus was observed at the center of the cell.

b) Treated with IBV 40-24 previously subjected to 56 C for 30 minutes: Infectious bronchitis virus-infected allantoic fluid was incubated at 56 C for 30 minutes (indicator virus) and was used for the HA test. Specimens were prepared for electron microscopy after 20, 40 and 60 minutes of exposure of the cells to the indicator virus. Attachment of the virus to cell was not observed in any of the specimens. Morphological changes of the cell membrane consisted of small depressions which were only present in the cells after 60 minutes, but not with cells after 20, and 40 minutes exposure (Figure 11).

c) Treated with trypsin-modified IBV 40-24 for 20 minutes: These cells resembled normal cells except for the presence of small particles on the cell membrane which were observed after sample was shadowcast. (Figure 12). The number of these particles was less than the number of virus particles attached to the nonhemolyzed cell.

d) Treated with trypsin-modified IBV 40-24 for 60 minutes: The changes of the cell were mostly small depressions on the membrane with a diameter of about 200 mμ. No virus was observed on the membrane (Figure 13). It seems that these depressions may have been caused by the virus on the cell membrane. With agglutinated, non-hemolyzed erythrocytes, the virus was released from the cells within 60 minutes. Small projections had been produced on the surface of the cell and were related to the attachment of the cells (Figures 6, 7, and 9). Comparing those projections to the depressions on the hemolyzed cells, it may be considered that the depressions present are either where the virus had been attached, or the bases where projections could have been present on the agglutinated cells.

The depressions were also observed on the membrane of the cells treated with indicator virus. The size of these depressions was, however, smaller than that produced by trypsin-modified virus.

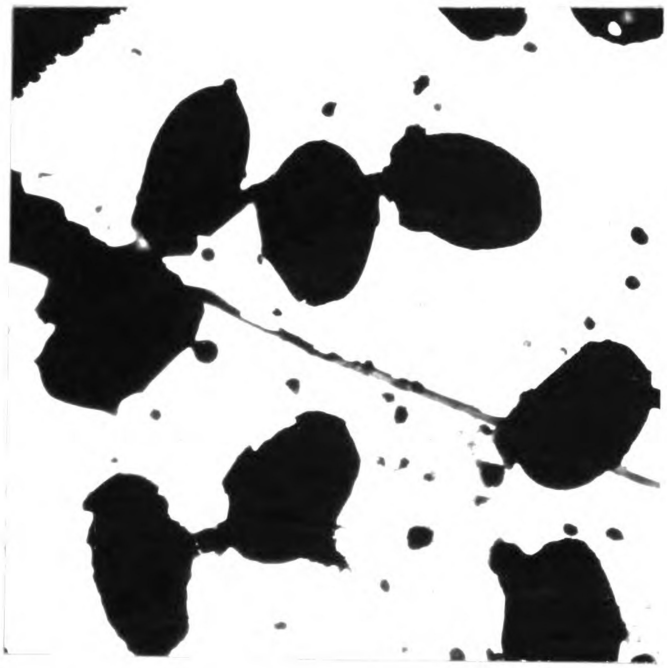

FIGURE 6. Electron micrograph of the agglutination pattern of chicken erythrocytes treated with trypsin-modified IBV 40-24. X 7600

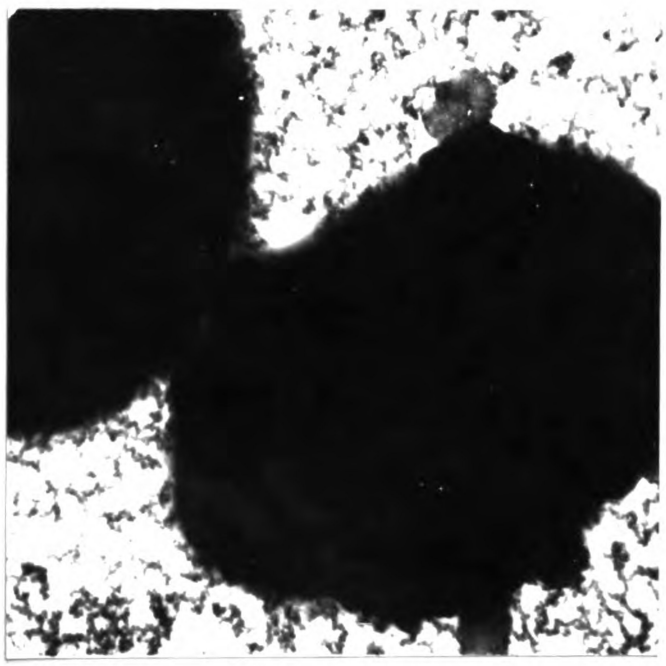

FIGURE 7. Electron micrograph of erythrocytes agglutinated by trypsin-modified IBV 40-24 after 60 minutes exposure, shadowcast. X 15200

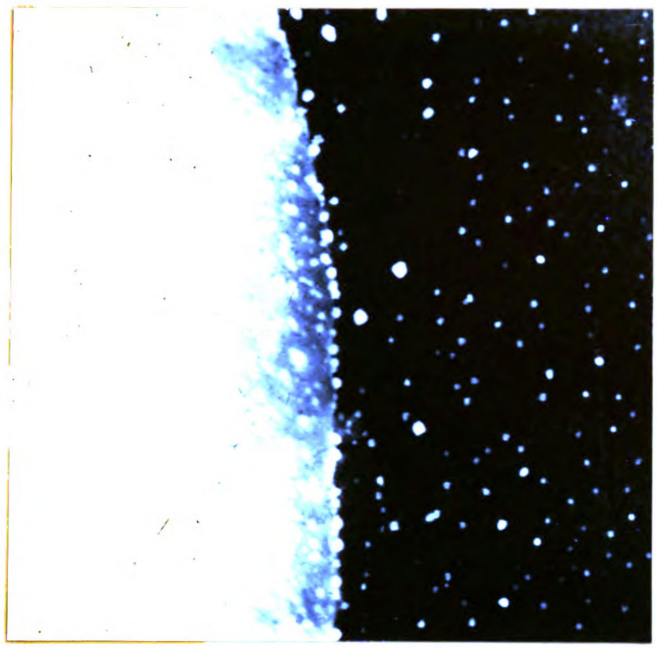

FIGURE 8. Electron micrograph of erythrocyte treated with trypsin-modified IBV 40-24 for 20 minutes. X 40000

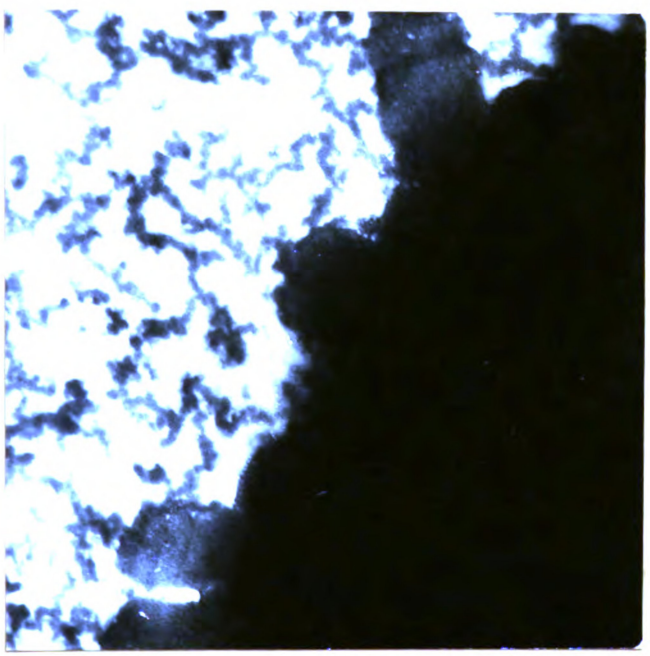

FIGURE 9. Electron micrograph of erythrocytes agglutinated by trypsin-modified IBV 40-24 for 60 minutes, shadowcast. X 22800

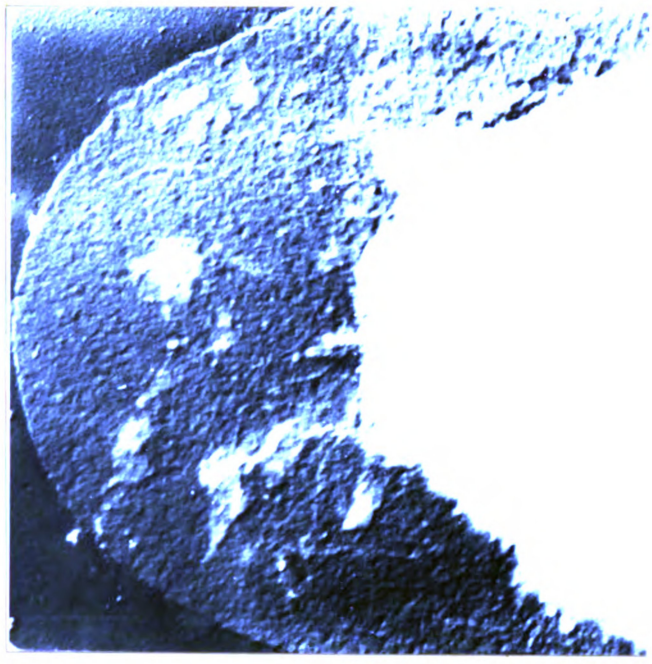

FIGURE 10. Electron micrograph of nontreated hemolyzed erythrocyte, shadowcast. X 22800

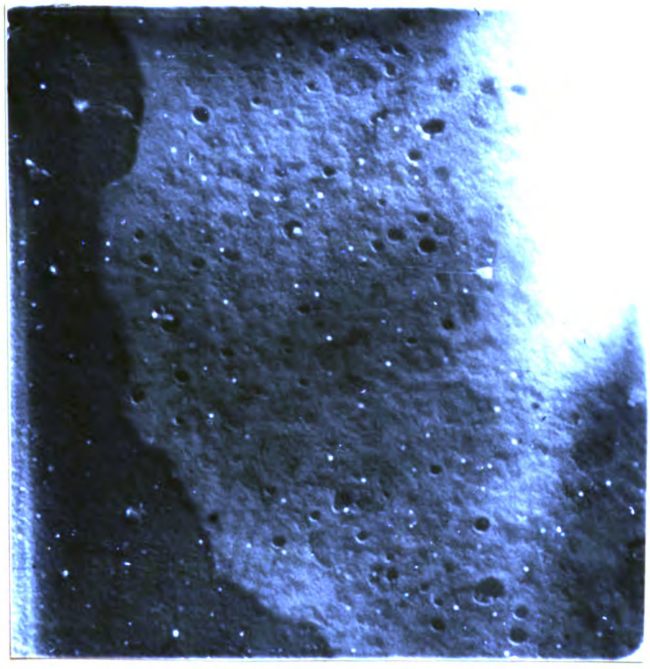

FIGURE 11. Electron micrograph of hemolyzed erythrocyte treated with indicator IBV 40-24 for 60 minutes, shadowcast. X 22800

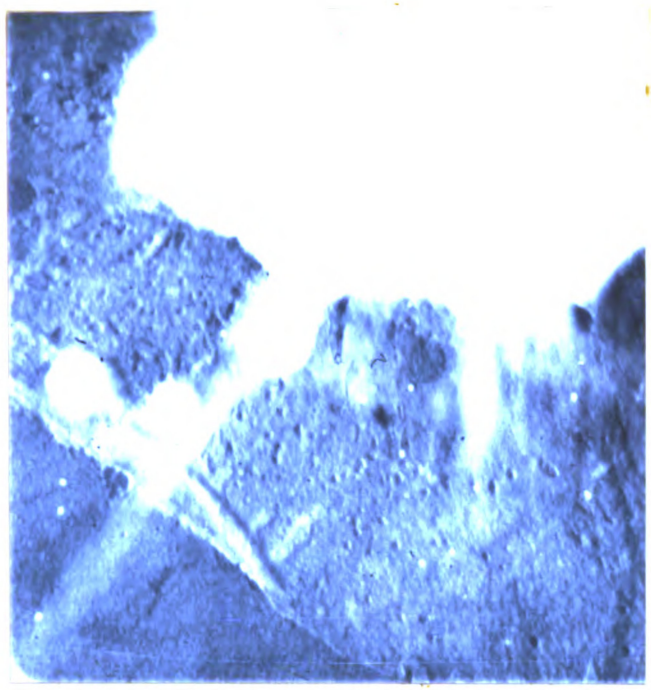

FIGURE 12. Electron micrograph of hemolyzed erythrocyte treated with trypsin-modified IBV 40-24 for 20 minutes, shadowcast. X 22800

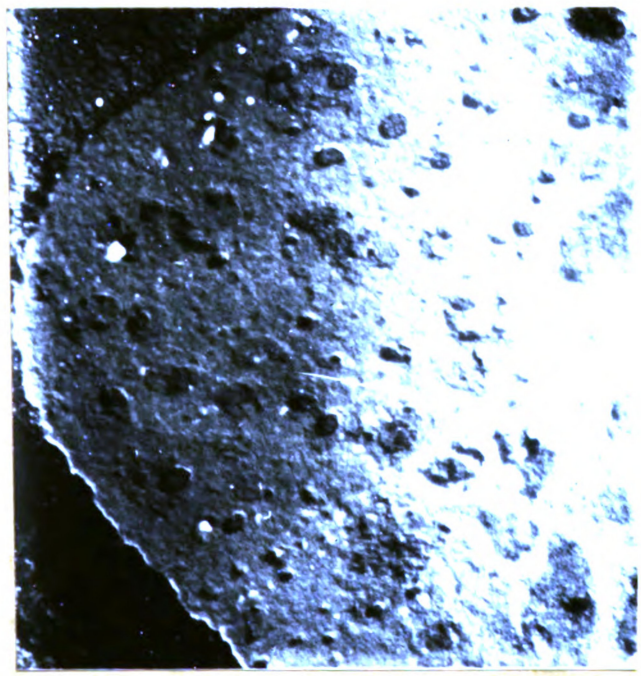

FIGURE 13. Electron micrograph of hemolyzed erythrocyte treated with trypsin-modified IBV 40-24 for 60 minutes, shadowcast. X 22800

DISCUSSION

Modification of infectious bronchitis virus by trypsin induces hemagglutination activity of the virus. The mechanism of this induction must be determined in order to understand the virus cell interaction and to establish standard HA and HI tests.

With the myxoviruses and some of the arthropod borne viruses, direct adsorption of the virus to the erythrocyte causes agglutination which is followed by enzymatic destruction of the cell receptor by the virus and elution of the virus from the cell. With some of the viruses of the pox group, hemagglutination is caused by a soluble antigen separable from the virus particle.

It was suggested in previous studies that the hemagglutinin of IBV is present in two fractions (Corbo and Cunningham, 1959), one related to the virus particle, and the other consisting of the greater part, distinct from the virus particle, and that the hemagglutinin, but not the infective particle is retained by filters of 50 mμ pore size (Muldoon, 1960). In chicken embryo culture, infectivity is at the maximum 24 hours after inoculation and the hemagglutinin is at the maximum at 72 hours. Based on infectivity tests in embryos, IBV does not sediment at 44000 g (Buthala, 1956). The virus adsorbs to

anion exchange resin and elutes from the resin subsequently in 10 per cent Na_2HPO_4 solution. Eluted virus showed a higher infectivity titer than the original virus (Mallmann, 1960).

Neither infectivity nor HA activity could be demonstrated from the pellet obtained by nine cycles of centrifugation at 136000 g. Viral particles in allantoic fluid after being partially purified by centrifugation at 21500 g for one hour were from 60 to 80 mμ. Particles from 20 to 40 mμ were also present in infected and normal allantoic fluid. Particles eluted from resin were more homogeneous in size and shape than those of infected allantoic fluid prior to treatment with resin and consisted almost exclusively of particles from 60 to 80 mμ. The number of virus aggregates was also less in the eluted virus than in the original virus. The increase in the infectivity titer of the eluted virus found by Mallmann (1960) is possibly due to the disaggregation of virus particles in the course of adsorption and elution of the virus. Adsorption to and elution from resin offers a possible means of separation of virus particles from particles ordinarily present in normal allantoic fluid, and for partial purification of the virus.

The hemagglutination titer of the eluted virus was the same as with the original virus. This indicates

that the hemagglutinin is not present in solution as a free antigen, but is rather related to the virus particles adsorbed to the resin. From the homogeneity of the eluted particles, it may be concluded that there is a similarity in the size and shape of infective and hemagglutinin particles even if they are, in fact, independent of each other.

Untreated virus, and virus eluted from resin, were reduced from 60 to 80 mμ to 25 to 35 mμ when modified with trypsin for 30 minutes at 56 C.

The activity of trypsin has been tested on a variety of peptides and proteins (Dixon and Webb, 1958) and has been found to be more specific than most of the proteolytic enzymes. There are known cases in which trypsin does not readily attack a bond at the carboxilic end of basic amino acids as would be expected. Two such bonds (one involving arginine and the other lysine) occur in tobacco mosaic virus (Woody and Knight, 1959). Induction of hemagglutination activity of IBV by tryptic digestion of the protein of the virus may indicate that arginine or lysine or both be present in the molecular structure of the virus (Corbo and Cunningham, 1959).

Since trypsin has specific activity on proteins, it may be concluded that a protein coat from 17 to 22 mμ thick is removed from the surface of the virus by trypsin. Influenza virus has an outer protein coat which is thought

(Hotchin et al., 1958) to be analogous to the cell membrane around the virus derived from the cell previously infected by the virus. Hemadsorption, whereby cells infected with influenza virus are able to adsorb erythrocytes, also indicates that the membrane of the cell possesses the same function as influenza virus as reflected by hemagglutination. It is possible that after infection of the cell, the virus behaves as a cytoplasmic gene, the presence of which is reflected as a phenotypic change in the host which can be detected by the ability of the host cell membrane to behave like the virus membrane. The protein coat of IBV might act similarly as an altered cell protein which inhibits the hemagglutinative activity of the virus, or as a mucoprotein inhibitor of HA which covers the surface of the virus. A mucoprotein present in the allantoic fluid does not mask the HA activity of the myxoviruses, but hemagglutination by IBV is not detectable until after the virus has been modified by trypsin.

Neither nonmodified IBV, nor indicator IBV were able to adsorb to the erythrocyte membrane. Trypsin-modified virus was adsorbed within 20 minutes after contact with the cell. After 60 minutes, projections were produced on the surface of the cell by the virus which was subsequently eluted from the cell. These projections acted as a lattice for attachment of other cells to cause

agglutination. With hemolyzed cells, small depressions of 200 mμ were observed instead of the projections from the normal intact cells. The depressions were probably where the virus had been attached to the cell or the bases where the projections would have been present on the agglutinated cells. No such morphological changes were observed on cells treated with trypsin alone or with trypsin and egg-white trypsin inhibitor.

SUMMARY

1. Infectious bronchitis virus in allantoic fluid is a sphere from 60 to 80 mμ in diameter.

2. The virus can be partially purified and separated from particles, 20 to 40 mμ, ordinarily present in normal allantoic fluid by centrifugation at 21500 g and by adsorption of the virus to anion exchange resin and subsequent elution in 10 per cent Na_2HPO_4 solution.

3. The hemagglutinin of IBV is not present as a soluble antigen in the supernatant fluid, but is related to the virus particle.

4. The morphological characteristics of the infective particle and hemagglutinin are similar.

5. A protein coat from 17 to 22 mu thick is present on the surface of the virus and is removed by trypsin at 56 C for 30 minutes.

6. Trypsin-modified IBV attaches to the erythrocyte membrane within 20 minutes.

7. The virus causes a few projections to be produced on the membrane of the normal erythrocyte and small depressions on the hemolyzed erythrocyte which is followed by elution of the virus from the cell.

BIBLIOGRAPHY

Ada, G. L., and Perry, B. T. (1955). Infectivity and nucleic acid content of influenza virus. Nature. 175:209-210.

Anderson, S. G. (1947). The reaction between red cells and viruses of the influenza group. Austr. J. Exp. Biol. Med. Sci. 25:163-174.

Anderson, S. G. (1959). Hemagglutination by animal viruses. The Viruses. Vol. III. Animal viruses, Edited by Burnet, F. M., and Stanley, W. M. 21-50.

Bateman, J. B., Davis, M. S., and McCaffery, P. A. (1955). A note on the reaction of heated influenza virus protein with red blood cells. Am. J. Hyg. 62: 349-354.

Beaudette, F. R., and Hudson, C. B. (1937). Cultivation of the virus of infectious bronchitis. J. Am. Vet. Med. Assoc. 90 (1):51-60.

Brandon, F. B. (1957). Ph.D. thesis, University of Pittsburgh, cited by Buzzell, A., and Hanig, M. (1959). The mechanism of hemagglutination by influenza virus. Advances in virus research. 5:289-340.

Burnet, F. M., and Anderson, S. G. (1946). Modification of human red blood cells by virus action. I. The erceptor gradient for virus action in human red cell. Brit. J. Exp. Path. 27:228-236.

Burnet, F. M. (1952). Hemagglutination in relation to host-virus interaction. Ann. Rev. Micro. 6:229-241.

Burnet, F. M., and Edney, M. (1952). Influence of ions on the interaction of influenza virus and cellular receptors or soluble inhibitors of them. Austr. J. Exp. Biol. Med. Sci. 30:105-118.

Buthala, D. A. (1956). Some properties of the avian infectious bronchitis virus. Ph.D. thesis, Iowa State College, Ames, Iowa.

Buzzell, A., and Hanig, M. (1958). The mechanism of hemagglutination by influenza virus. Advances in virus research. 5:289-340.

Chomiak, T. W., Luginbuhl, R. E., and Jungherr, E. L. (1958). The propagation and cytopathogenic effect of an egg-adapted strain of infectious bronchitis virus in tissue culture. Avian Dis. 2:456-465.

Corbo, L. J., and Cunningham, C. H. (1959). Hemagglutination of trypsin modified infectious bronchitis virus. Am. J. Vet. Research. 20:876-883.

Cunningham, C. H., and Stuart, H. O. (1946). The effect of certain chemical agents on the virus of infectious bronchitis of chickens. Am. J. Vet. Res. 7:466-469.

Cunningham, C. H., and Stuart, H. O. (1947). Cultivation of the virus of infectious bronchitis of chickens in embryonating chicken eggs. Am. J. Vet. Res. 8:209-212.

Cunningham, C. H., and Stuart, H. O. (1947 a). The pH stability of the virus of infectious bronchitis of chickens. Cornell Vet. 37:99-103.

Cunningham, C. H., and El Dardiry, A. H. (1948). Distribution of the virus of infectious bronchitis of chicken in embryonated chicken eggs. Cornell Vet. 38:381-388.

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

Cunningham, C. H. (1960). Recent studies on the virus of infectious bronchitis. Am. J. Vet. Res. 21: 498-503.

Delaplane, J. P., and Stuart, H. O. (1939). Studies on infectious bronchitis. R.I. State Agr. Exp. Sta. Bull. 273-281.

Dixon, M., and Webb, C. E. (1958). Enzymes, 242-298. Academic Press, New York.

Domermuth, C. H., and Edwards, O. F. (1954). Electron microscope observation of thin sections of chorioallantoic membrane infected with infectious bronchitis virus. Proc. Soc. Am. Bact. 71-72.

Donald, H. B., and Isaacs, A. (1955). Some properties of influenza virus filaments shown by electron microscopic particle count. J. Gen. Microbiol. 11:325-331.

Donald, H. B., and Isaacs, A. (1954). Counts of influenza virus particles. J. Gen. Microbiol. 10:457-465.

Dounce, A. L., Howland, J. W., and Lan, T. H. (1943). Isolation and properties of chicken erythrocyte nuclei. Saina 97:584.

Dowson, I. M., and Elford, W. J. (1949). The investigation of influenza and related viruses in the electron microscope by a new technic. J. Gen. Microbiol. 3:298-301.

Edney, M. (1949). The influence of calcium ion on Vibrio cholera enzyme RDE and influenza virus with specific mucopolysaccharides. Austr. J. Exp. Biol. Med. Sci. 33:196-201.

Fabricant, J. (1949). Studies on diagnosis of Newcastle disease and infectious bronchitis. IV. The use of serum neutralization test in the diagnosis of infectious bronchitis. Cornell Vet. 41:68-80.

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

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

Friedman, M., and Bennett, C. R. (1957). A hemagglutination test for detection of adeno virus antibodies. Proc. Soc. Exp. Biol. Med. 94:712-717.

Hanig, M. (1948). Electrokinetic change in human erythrocytes during adsorption and elution of PR8 influenza virus. Proc. Soc. Biol. Med. 68:385-392.

Henle, G., Girard, A., and Henle, W. (1955). A nontransmissible cytopathogenic effect of influenza virus in tissue culture accompanied by formation of noninfectious hemagglutinins. J. Exp. Med. 101:25-41.

Hirst, G. K. (1941). The agglutination of red cells by allantoic fluid of chicken embryos infected with influenza virus. Science, 94:22-23.

Hirst, G. K. (1942). Adsorption of influenza virus hemagglutinins and the virus by red blood cells. J. Exp. Med. 76:195-209.

Hitchner, S. B., and White, P. G. (1955). Growth curve studies of chick embryo propagated infectious bronchitis virus. Poultry Sci. 34:590-594.

Hofstad, M. S. (1956). Stability of avian infectious bronchitis virus at 56 C. Cornell Vet. 46:122-128.

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

Hotchin, J. E., Cohen, S. M., Ruska, H., and Ruska, C. (1958). Electron microscopical aspects of hemadsorption in tissue cultures infected with influenza virus. Virology 6 (3):679-700.

Hoyle, L. (1950). The multiplication of influenza virus in fertile egg. J. Hyg. 48:180-188.

Hoyle, L. (1952). Structure of influenza virus. The relation between biological activity and chemical structure of virus fraction. J. Hyg. 50:229-245.

Hoyle, L., Jolles, B., and Mitchell, R. G. (1954). The incorporation of radioactive phosphorus in the influenza virus and its distribution in serologically active virus fraction. J. Hyg. 52:119-127.

Isaacs, A., and Donald, H. B. (1955). Particle counts of hemagglutinating viruses. J. Gen. Microbiol. 12:241-247.

Lauffer, M. A., and Carnelly, H. L. (1945). Thermal destruction of influenza A virus hemagglutinin. I. The kinetic process. Arch. Biochem. 8:265-274.

Lennet, E. H. (1959). Serological reactions in rikettsial and viral infections. Viral and rikettsial infections of man. Third edition, Edited by Rivers, Th. M., and Horsfall, F. L. 230-250. J. B. Lippincott Company, Philadelphia.

Levine, S., Puck, T., and Sagik, B. P. (1953). Absolute assay for virus hemagglutination. J. Exp. Med. 98:521-531.

Loomis, L. M., Cunningham, C. H., Gray, M. L. and Throp, F. (1950). Pathology of the chicken embryo infected with infectious bronchitis virus. Ann. J. Vet. Res. 11:245-251.

McClelland, L., and Hare, R. (1941). The adsorption of influenza virus by red cells and a new invitro method of measuring antibodies of influenza virus. Canad. Public Health J. 32:530-538.

Mallmann, V. H. (1960). The influence of anionic resin exchanges on infectious bronchitis virus cultivated on embryonating chicken eggs and whole chicken embryo cultures. Ph.D. thesis, Michigan State University, East Lansing, Michigan.

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

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

Paucker, K., Brich-Anderson, A., and von Magnus, P. (1959). Studies on the structure of influenza virus. I. Components of infectious and incomplete particle. Virology, 8 (1):1-20.

Porterfield, B. M. (1952). The effect of calcium and electrolytes on the enzymatic action of influenza virus and Vibrio cholera extract. Brit. J. Exp. Path. 33:196-201.

Reagan, R. L., Hanser, J. E., Lillee, M. G., and Corigi, A. H. (1948). Electron microscope study of the virus of infectious bronchitis in chicken. Cornell Vet. 38:190-191.

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

Reed, L. J., and Muench, H. (1938). A simple method of estimating fifty per cent end points. Am. J. Hyg. 27:493-499.

Sagik, B. P., and Levine, S. (1957). The interaction of Newcastle disease virus with chicken erythrocytes. Attachment, elution, and hemolysis. Virology, 3:401-416.

Schafer, W. (1956). Units isolated after splitting fowl plague virus. Ciba Foundation Symposium on the Nature of Viruses. Edited by Wolstenholme, G. E., and Miller, E. C. P. 91-115. Little, Brown and Company, Boston.

Schlesinger, R. W. (1950). Incomplete growth cycle of influenza virus in mouse brain. Proc. Soc. Exp. Biol. Med. 74:541-548.

Scott, L. E., Felton, F. G., and Barney, J. A. (1957). Hemagglutination with herpes simplex virus. J. Immunol. 78:211-213.

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

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

Stone, J. D. (1949). Inhibition of influenza virus hemagglutinin by mucoids. Conversion of virus to indicator for inhibition. Aust. J. Exp. Biol. Med. Sci. 27:337-352.

Tyrrell, D. A., and Valentine, R. C. (1957). The assay of influenza virus particles by hemagglutination and electron microscopy. J. Gen. Microbiol. 16:668-675.

von Magnus, P. (1951). Propagation of the PR8 strain of influenza A virus in chicken embryos. II. The formation of "incomplete" virus following inoculation of large doses of seed virus. Acta. Pathol. Microbiol. Scand. 28:278-293.

van Kooyen, G. E. (1954). A revision of Holme's classification of animal viruses. Suborder III. (Zoophagineae). Canad. J. Microbiol. 1:227-284.

Woese, C. R. (1956). Heat inactivation studies on animal viruses. I. The inactivation of virus hemagglutinin. Arch. Biochem. Biophys. 63:212-220.

Woody, B. R., and Knight, C. A. (1959). Peptide map obtained with tryptic digests of the proteins of some strains of tobacco mosaic virus. Virology, 6 (3):689-701.

Wright, B. S., and Sagik, B. P. (1958). Plaque formation of monolayers of chicken kidney cells. Virology, 5:573-574.