SOME CHARACTERISTICS OF THE HEMAGGLUTINATING ACTIVITY OF INFECTIOUS BRONCHITIS VIRUS

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SOME CHARACTERISTICS OF THE HEMAGGLUTINATING ACTIVITY OF INFECTIOUS BRONCHITIS VIRUS

By

ROBERT LEE MULDOON

A THESIS

Submitted to the School for Advanced Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

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1960

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This thesis is

respectfully dedicated

to

MY FAMILY

ABSTRACT

The common method for titration of infectivity of infectious bronchitis virus and its antibody uses the embryonating chicken egg as the indicator. Hemagglutination by this virus can be demonstrated after it has been modified by trypsin.

The present study deals with certain properties of the trypsin modified hemagglutinin of infectious bronchitis virus with the primary purpose of development of a hemagglutination-inhibition test.

The hemagglutination titers obtained after 30 minutes incubation with trypsin at 56 C are comparable to those obtained after three hours incubation at 37 C. Eggwhite trypsin inhibitor is added after incubation. The maximum hemagglutination titer is obtained when one percent trypsin and one percent eggwhite trypsin inhibitor is used.

Trypsin modified virus can be stored at -65 C for three weeks without loss of hemagglutinating activity. Chicken erythrocytes could not be stored in Alsever's solution for longer than one week and be used in the hemagglutination test.

The only reactive erythrocytes for hemagglutination of IBV are those from chickens and turkeys. Cells from chickens three weeks or older are reactive, but maximum

activity is observed from birds five weeks or older.

The hemagglutinin prior to trypsinization of infectious bronchitis virus is stable at 56 C for at least six and one-half hours, whereas infectivity is lost within two hours. Antigenicity as detected by serum neutralization tests is retained for two hours at 56 C. The infectivity of the virus decreases significantly after incubation with trypsin at 37 C.

Analysis of viral multiplication and hemagglutinating activity reveals that the hemagglutination titer is maximal at 72 hours post inoculation whereas infectivity is highest at 24 hours. No decrease of infectivity is observed after reaction of the trypsinized virus with chicken erythrocytes. Filtrates from a 50 millimicron filter are infectious, but hemagglutinating activity is not demonstrated.

The activity of the receptor destroying enzyme of Vibrio cholerae is of the same magnitude when tested with influenza strain PR8 or infectious bronchitis virus 17-40.

Extracellular fluid from virus infected chicken embryo kidney cell culture gives the same hemagglutination titer as non-infected fluid. Hemadsorption is not observed prior to or after trypsinization.

The development of a usable hemagglutination-inhibition test probably depends on the removal of inhibitors of hemagglutination which are present in negative and anti-infectious bronchitis virus sera. Heat or filtration does

not remove inhibitors. Trypsin, periodate, butanol, ether, zymosan or the receptor destroying enzyme of Vibrio cholerae does not influence the action of serum inhibitor. When trypsin treated serum was subsequently reacted with periodic acid, all inhibitors are destroyed.

ACKNOWLEDGMENTS

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INTRODUCTION

Infectious bronchitis virus and its neutralizing antibody are generally assayed using embryonating chicken eggs as the indicator host of viral infectivity.

Infectious bronchitis virus will not agglutinate erythrocytes, but when the virus is treated with trypsin hemagglutination occurs.

The present studies are concerned with the characteristics of some of the properties of the agglutinating agent with the primary purpose being the possible development of a hemagglutination-inhibition test.

LITERATURE REVIEW

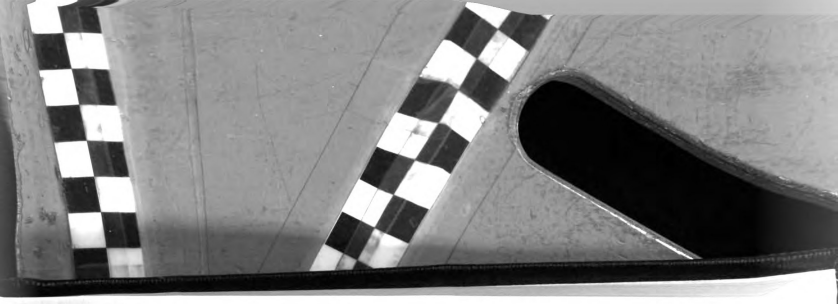
The virus of infectious bronchitis of chickens is classified as Tarpeia pulli by van Rooyen[119]. This disease, first described by Schalk and Hawn[102] in 1931, is limited to chickens.

Infectious bronchitis virus (IBV) as determined by electronmicroscopy is a sphere ranging from 60 to 100 millimicrons[91,92]. Filamentous projections are present in specimens prepared in saline or in distilled water, but they are more prominent in the former. These filaments have not been detected in the infected chorioallantoic membrane where the elementary bodies have a mean diameter of 200 millimicrons[31].

The infected tracheal mucosa of the chicken undergoes cyclic changes of acute, reparative, and immune phases for 18 to 21 days[70].

The embryonating chicken egg is used as the indicator host for assay of infectivity of IBV and its antibody. Serial passage of IBV in the embryonated egg results in an increase of virulence for the embryo and a loss of antigenicity for the chicken[5,30].

Viral activity in eggs can be detected by macroscopic as well as microscopic alterations. Pathognomonic lesions of the embryo are caused by early egg passage virus. "Curling" of the embryo consists of a wry neck and deformed



feet compressed over the head. Embryos may be dwarfed as much as one half size with a firm, ball like shape[5,30,33,78]. Another indication of viral infectivity is the presence of urates in the mesonephros[78].

Some microscopic changes in virus infected cells are edema of the chorioallantoic membrane; hepatic hemorrhage, necrosis, and abcess formation; and interstitial nephritis and necrosis. Inclusion bodies have not been observed[78].

Embryo mortality is not a constant finding in the early egg passages of IBV. Cunningham and Jones[26] observed the greatest direct correlation between embryo mortality and number of egg passage when eggs were inoculated via the allantoic cavity. Although this virus may be cultivated using several routes of inoculation, the allantoic cavity is the route of choice because of simplicity of operation and amount of infected material recovered.

Singh[104] found that egg propagated IBV has a diphasic characteristic and a bimodal rate of inactivation at 56 C which is indicative of a three-halves order reaction. At room temperature, virus infected allantoic fluid remains viable for five to seven days[88].

Inactivation of this virus is achieved within three minutes by certain chemicals, of which some are, one percent phenol, one percent metaphen, 90, 70, 40, and 25 percent ethanol and one percent formalin[27].

Infectious bronchitis virus is more stable at 4 C

in an acid medium than in an alkaline medium for 60 days. However, the reverse is true from 60 to 170 days with an optimum pH of 7.80. The isoelectric point is probably about pH 4.05[28].

Buthala[19] determined the density of IBV to be approximately 1.15 with minor variations depending on the solvent. He was unable to concentrate the virus by centrifugation at 40,000 g for two hours.

Infectious bronchitis virus does not possess hemagglutinating activity detectable by the usual methods[34,65]. Corbo and Cunningham[25] have shown thar virus-infected allantoic fluid, to which trypsin and eggwhite trypsin inhibitor (ETI) have been added, contained an active hemagglutinating agent.

Hemagglutination

Erythrocytes may be agglutinated by extracts of plant[86] and animal tissues[105], the higher fungi[35], bacteria[86] rickettsiae and pluropneumonia-like organisms[35] as well as viruses[57]. Hemagglutination (HA) by viruses offers a tool for analysis of the virus-cell interaction and definition of some biochemical properties of the virus. It may also serve as a possible basis for the classification of viruses.

Viral hemagglutination, and the inhibition of this reaction by specific antibody, was first reported by

Hirst[57] in 1941. Working independently, McClelland and Hare[79] also reported on viral HA, hemagglutination-inhibition (HI), and the species of cells that may be used to characterize the reaction.

Viruses which participate in HA have been classified into four groups; 1) myxoviruses, 2) arthropod-borne viruses, 3) pox viruses, and 4) viruses which cannot be grouped into the first three categories[63].

The myxoviruses[4] are those of influenza, Newcastle disease, fowl plague, and mumps. Basic research on the mechanism of HA of this group has been done with the PR8 strain of influenza as the prototype. The members of this group possess the ability of splitting neuraminic acid derivatives from mucoproteins which are present in body fluids and on the surface of erythrocytes[63]. The hemagglutinin is part of the virus particle[58,59].

The sequence of reaction is adsorption of the virus onto the receptor sites on the cell, agglutination, and finally elution of the virus from the cell after destruction of the receptors.

At the time of adsorption of the virus to the cell, the reaction is reversed by decreasing the salt concentration of the diluent[112]. Adsorption may occur over a wide virus-cell ratio. The temperature may range from 4 C to 37 C. Physical data indicate that the initial attachment is ionic whereas chemical data suggest that the attachment occurs

through the hydroxyl group on the substrate[20].

Adsorption can be prevented[15] or limited[40] by control of the ionic concentration, presumably by reducing the mutual replusion of the virus and of the cell. The HA titer of PR8 is constant within the range of pH 6.0 to 8.0[83]. The HA of Newcastle disease is optimal within the range of pH 5.8 to 6.2, although titers were obtained from pH of 5.0 to 9.0[97].

The second event in the sequence is agglutination of the cells. Isaccs and Edney[68], and Schlesinger and Karr[103] suggest that the hemagglutinin of the myxoviruses is polyvalent, and forms a lattice of cells.

The final process is the elution of the virus from the cell. Elution is dependent upon the presence of cations of which calcium is the most efficient[15]. Sodium hexametaphosphate adversely affects the degree of elution probably by negation of the effect of the calcium ion[89]. Active virus can destroy the major portion of the receptors on a cell without becoming detached from it[12,53,110]. Sagik[98] suggested that the virus is fixed in one location and causes a rippling disturbance on the remainder of the cell surface. Burnet[12] proposed that the virus migrates over the cell surface and in so doing destroys the receptors. Regardless of the mechanism, viruses can agglutinate fresh cells repeatedly without loss of activity. Cells which were acted upon by the virus were not reagglutinable by the same virus.

Hirst[57] had previously suggested that the HA mechanism was enzymatic in nature. Cells after agglutination with a given virus may or may not be agglutinated by another virus. For example, cells which have been acted upon by mumps will not be agglutinated by influenza strain MEL. Cells agglutinated by MEL will be agglutinated by mumps. Such a series constitutes a "receptor gradient" as described by Burnet[17], and adds supplemental support to Hirst's enzyme hypothesis.

The final proof of an enzyme reaction is the isolation and purification of the enzyme and substrate. As a corollary, the cleavage products should be identified and the kinetics of the reaction studied. The components of the virus responsible for infectivity, HA, and elution have not been separated by physical means[51,117]. Hoyle[67] was able to disintegrate influenza particles with ether with the subsequent release of two fractions, one of which carried HA activity.

Inhibitors of HA activity have been found in many biological materials one of which is urine. Tamm and Horsfall[114] were able to purify this inhibitor. When an electrophoretically homogenous mucoid was incubated with purified influenza virus type B, enzymatic cleavage occurred[47]. Klenk[71] identified the end product of this reaction as N-acetylneuraminic acid.

Inhibitors

There are three major inhibitors of viral hemagglu-

tination. The first is specific antiserum which is produced in response to the hemagglutinin proper or to the infective virus particle. It is stable at 62 C for 30 minutes, and is not destroyed by the receptor destroying enzyme (RDE) of Vibrio cholerae, or moderate concentrations of periodate[69].

The second inhibitor is the thermolabile Chu inhibitor[80], present in serum, which will at times reduce viral infectivity. It is precipitated with gamma globulin, and is destroyed by RDE[3] only if the inhibitor preparation is crude[22]. Inactivation occurs in the presence of trypsin[116], but the action of periodate is debatable[22,60].

The third inhibitor of HA activity is a mucopolysaccharide found in many normal biological materials, such as serum[42], red blood cells[43], allantoic fluid[118], and human urine[113]. The initial work was done with the serum mucoid.

Stone[108] observed that when influenza virus was heated at 56 C, the ability of the virus to elute from cells was destroyed. This was named an indicator virus. Certain strains required specific conditions before conversion occurred. Indicator virus may be produced by the action of trypsin[109]. Incubation with one mg per ml of potassium periodate will yield an indicator virus, but HA activity is destroyed when eight mg per ml of periodate is used[39].

Francis[42] observed that the HI titers obtained with indicator viruses have no correlation with specific antibody

as determined by neutralization tests. Indicator virus has no effect on the inhibitor, which is destroyed by an active virus[9,60].

Burnet[16] found that this serum inhibitor precipitated with the globulin fraction and is heat stable. Hirst[60] noted a marked reduction in inhibitory titer of normal rabbit serum after incubation with trypsin. The non-specific inhibitor of HA of chicken erythrocytes by mumps and Newcastle disease is eliminated by treating the serum with trypsin and periodate[121]. The inhibitor of influenza virus in allantoic fluid can be destroyed by trypsin[55] and periodate[77]. Lanni and Beard[74] destroyed the eggwhite inhibitor with dilute periodate. Inhibitors of some arthropod-borne viruses can be removed by filtration of the serum[21]. Normal serum may be dialysed[122] or treated with sodium citrate[29] to remove the inhibitor of certain myxoviruses.

Burnet[18], and Friedewald *et al.*[43] showed that the inhibitor described by Francis is similar to the receptor substance of intact erythrocytes to which the virus attaches to produce HA.

Capsular material from pneumococci[23] and a toxin from *Clostridium welchii*[81] exhibit the same effect as the viral enzyme in that red cells are rendered inagglutinable. Anderson *et al.*[3] demonstrated that the "Francis inhibitor" is destroyed by RDE from *V. cholera*. It is unknown whether this latter enzyme preparation is homogeneous[18].

The activity of RDE is optimal at pH 6.2, and calcium is required[32]. The activity is destroyed after 30 minutes at 52.5 C in the absence of calcium, but is stable for 60 minutes in the presence of M/100 calcium[2]. RDE is destroyed by crystalline trypsin at pH 7.2 to 8.5[10,109]. The electrophoretic mobility of the cell is decreased after the action of RDE[2] or more active virus[90], indicating that a new surface antigen may be produced or uncovered by the action of the RDE[13]. All mucoid inhibitors which inhibit the HA of indicator viruses are rendered less active by RDE[10,62,109]. If the mucoid is in solution, complete destruction occurs[11]. If RDE is administered to mice prior to infection by influenza virus, the susceptible tissue is resistant to infection, probably due to the stripping off of receptor site on the cells[107].

The action of active virus and RDE on mucoid inhibitors is the same in all cases but two. According to Tamm and Tyrrell[115], RDE has no effect on the inhibitor in normal allantoic fluid and human urine which affect the HA activity of murine encephalomyelitis GD VII and certain influenza strains. By starch zone electrophoresis, it has been shown that it is chemically distinct from the LEE influenza B inhibitor. The inhibitor of Newcastle disease virus, in allantoic fluid, is not destroyed by this enzyme.

Although all inhibitors which have been studied extensively are mucopolysaccharide, certain differences

are observed. The degree of inhibition of HA by active virus varies with the source of the inhibitor[8]. If an inhibitor which had been degraded by active virus was subsequently tested with an indicator virus, a receptor gradient was established showing characteristic differences[109]. The action of periodate differs with various inhibitors[9,37].

In an attempt to analyse the composition of the substrate for the viral enzyme, inhibitors from various sources were isolated and studied. The urinary inhibitor purified by Tamm and Horsfall[113] has a molecular weight of 7×10^6. It is soluble in water but insoluble in 0.85 N NaCl, and contains 20 percent carbohydrate by weight. Hexoses, hexosamines, and 2-carboxy-pyrrole appear in equal amount[83]. It is destroyed by active influenza virus and the receptor destroying enzyme of *V. cholerae*[18]. The product of the reaction of highly purified influenza B virus on electrophoretically homogenous urine mucoprotein or ovamucin[47] resembles N-acetylglucosamine. On further analysis, Klenk[71] identified the end product of the reaction as N-acetylneuraminic acid.

Green and Wooley[52] reported that HA of PR8 strain of influenza was inhibited by the presence of an extract obtained by heating chicken red blood cells at 100 C for five minutes. Friedewald *et al.*[43], using a Waring blendor, extracted an inhibitor from chicken cells. This inhibitor was rapidly inactivated at 65 C. An extract obtained when

dry ether was used was stable for two hours at 100 C[66]. Other extractions were performed using ether, chloroform and acetone in sequence[85]. McCrea[82] idolated an inhibitor using a serial extraction method of pentane and ethanol followed by cyclic freeze-drying. Trypsin had no effect on this inhibitor, but 0.005 M periodic acid destroyed it. Hirst[61] found that 0.001 M periodate could completely destroy the receptors of a 1.5 percent suspension of cells.

Sialic acid, the group name for ON-diacetylneuraminic acid, N-acetylneuraminic acid, N-glycolylneuraminic acid, and other acylated neuramic acids[6] had been found in all inhibitory mucoproteins[48,87]. Klenk and Lempfrid[72] were able to isolate N-acetylneuraminic acid from human erythrocytes after reaction with RDE. Using treatment with certain bases, Gottschalk[50] isolated N-acetylneuraminic acid linked ketosidically to N-acetylgalactosamine from bovine submaxillary gland mucoprotein[49]. Enzymatic action of influenza will hydrolyse the disaccharide into these two components. Neuraminidase is the name given to this supposed viral enzyme. The action of RDE yields the same products as the viral enzyme[50].

The second group of viruses which have been classified on the basis of the HA mechanism are the arthropod-borne viruses, which are the typical encephalitic viruses. The virus-cell reaction requires exacting conditions for demonstration of HA activity. West Nile virus will only

react with cells from chicks not older than 12 hours and from sheep at pH 7.5[95]. The hemagglutinin is thought to be associated with the virus particle[96]. There is no enzyme found as with the myxoviruses, and RDE has no effect on the cellular receptor site.

All known inhibitors of HA by this group of viruses can be destroyed by ether, chloroform, benzene, acetone[95], or Sietz filtration[21]. The inhibitors are stable at 100 C and not affected by potassium periodate[95].

The third group of viruses includes those of psittacosis and the pox diseases. They are characterized by a hemagglutinin distinct from the viral particle. Vaccinia has two hemagglutinins which are separable by centrifugation[44]. In extracts from the chorioallantoic membrane, there is no differentiation in activity. The agent first sedimented in centrifugation is not destroyed at 70 C, whereas the other is destroyed at 56 C within 45 minutes[24]. Both hemagglutinins are phospholipid, and are inactivated by lecithinase types A and C[105].

The hemagglutinin of mouse meningopneumonitis[56] and feline pneumonia[46] viruses is smaller than the infective particle.

The fourth group of viruses includes those of encephalomyocarditis, which agglutinate only sheep cells, the pneumonia virus of the mice which agglutinates only mouse cells and the polyoma virus of mice. With these viruses,

the hemagglutinin is believed to be associated with the viral particle, but there is no enzymatic destruction of receptor sites. There is a question whether this group is distinct from the arthropod-borne viruses.

The GD VII strain of Theiler's murine encephalomyelitis virus agglutinates human group O cells at 4 C, but not at 37 C[73]. After treatment with trypsin, HA activity can be demonstrated at 22 C[84]. Periodate and RDE have no effect on the cell receptors[36]. After 30 minutes at 56 C, there was a 99 percent reduction of HA activity[73].

Corbo and Cunningham[25] demonstrated that trypsin can induce HA of IBV. The rate and degree of adsorption of the hemagglutinin varied directly with temperature. Elution varied indirectly with temperature. The HA activity was inhibited by negative and anti-IBV serum, ovomucoid, and normal and virus-infected allantoic fluid. It was concluded that the hemagglutinin was probably present in two fractions; one in close association with the virus particle, and the second, which is in the majority, was distinct from the viral particle.

MATERIAL AND METHODS

Virus

The strains of IBV used were obtained from the North Central Repository at Michigan State University, and were identified as follows:

Repository Code	Source
3	M. S. Hofstad, (104), Iowa State College, Ames, Iowa. 1947.
16	Q. Hipolito, (66), Universidade Rural de Minas Gerais, Escola Superior de Veterinaria, Brazil. 1955.
17	F. R. Beaudette, (B-13-25), New Jersey Agricultural Experiment Station.
18	C. S. Roberts, (1005-7), Alabama Department of Agriculture.
40	C. H. Cunningham, (586), Michigan State University. 1956.
41	H. Van Roekel, University of Massachusetts. 1941. (291st Bird Passage).
42	F. R. Beaudette, (B 588), Rutgers University, New Brunswick, New Jersey. 1935. Egg Adapted.

The strains of IBV will be identified by the Repository Code Number and the number of egg passage; e.g. 3-16 indicates strain 3 in the 16th egg passage.

Embryonating chicken eggs, nine-to-eleven days old, were used for the cultivation of all strains of IBV. The

standard inoculum was 0.1 ml per egg. Strain 42, which is completely egg adapted, kills all embryos within approximately 36 hours. Strains in earlier egg passages do not consistently kill embryos. The allantoic fluid was usually harvested 72 hours after infection, pooled and stored at -30 C in screw cap vials. Bacteriological sterility tests were performed. Strains 41 and 42 from chicken embryo kidney cell cultures, prepared by M. Spring, were also studied for induction of HA.

At the time of use, the virus was thawed at room temperature, and centrifuged at 3,000 rpm for 10 minutes. The supernatant fluid was tested for studies of HA.

Reagents

A one percent suspension of Bacto trypsin (1:250) in distilled water was incubated at room temperature for 15 minutes, and then passed through a Seitz EK filter. The filtrate was dispensed into screw cap vials, and stored at -30 C. The final concentration of this enzyme suspension is unknown due to the amount of adsorption on the filter.

A one percent solution of eggwhite trypsin inhibitor (ETI) in distilled water was prepared and stored at -30 C. The solution was not filtered due to loss of activity.

The receptor destroying enzyme is obtained in lyophilized form from Behringwerke, Marburg-Lahn, Germany.

It is reconstituted in distilled water and diluted in calcium borate buffered saline. To 12 X 75 mm test tubes, 0.25 ml of one percent chicken red blood cells is added to equal volumes of diluted enzyme, and incubated at 37 C for 30 minutes. Ten HA units of test virus is added to the RDE red blood cell mixture, and incubated at 37 C for 30 minutes. The end point is the lowest dilution showing partial agglutination.

Diluents

Bacto hemagglutination buffer was the commonly employed diluent in the HA test. This buffer consists of 766 ml of M/15 Na_2HPO_4 and 233 ml of M/15 KH_2PO_4 solution. The final pH is 7.3.

The diluent used for infectivity titrations was Difco Nutrient broth.

Earle's solution was used to prepare dilutions of the virus infected allantoic fluid for HA and infectivity titrations in the experiments using an ultrafilter because the action of Bacto HA buffer on infectivity was unknown.

The ingredients of Earle's solution are:

Grams/liter		Grams/liter	
NaCl	6.80	$NaH_2PO_4 \cdot H_2O$	0.14
KCl	0.40	$NaHCO_3$	2.20
$CaCl_2$	0.20	Glucose	1.00
$MgSO_4 \cdot 7H_2O$	0.20		

The $CaCl_2$ was autoclaved separately to prevent its precipitation from the solution.

Calcium borate buffered saline was used for the dilution of RDE. The ingredients are:

	Grams/liter
$CaCl_2$	1.000
NaCl	9.000
H_3BO_3	1.203
$Na_2B_4O_7 \cdot 10H_2O$	0.052

The final pH should be 7.1 to 7.3.

Erythrocytes

Erythrocytes, obtained by cardiac puncture from Single Comb White Leghorn chickens, were the standard cells for the HA test.

Erythrocytes from certain fowl, for the determination of cell spectrum, were collected at the Gull Lake Bird Sanctuary. Cells from turkeys and mammals were obtained at the College of Veterinary Medicine, Michigan State University.

The blood was collected in test tubes containing 1 ml of a two percent sodium citrate solution for each six ml of blood. The cells were washed three times by centrifugation at 1,800 rpm in 0.85 percent saline. After the final washing, the saline was removed, the packed cells were stored at 4 C, and used within five days.

For storage of erythrocytes, Alsever's solution was used which consists of the following:

2.05	grams of dextrose
0.80	grams of sodium citrate
0.42	grams of sodium chloride
0.055	grams of citric acid
100.00	ml of distilled water

The ingredients were mixed, the pH was adjusted to 6.1, the solution was passed through a Seitz EK filter, placed in a sterile flask, and stored at 4 C.

Methods

The procedure, as described by Corbo and Cunningham[25], for the HA test was the addition of one ml of one percent trypsin to two ml of virus infected allantoic fluid, and incubated for three hours at 37 C in a water bath. Upon removal, one ml of ETI was added.

Serial two fold dilutions of the treated virus were prepared in buffer. To 12 X 75 mm tubes was added 0.25 ml each of the buffer, diluted virus and a 0.5 percent suspension of chicken erythrocytes. The control contained 0.5 ml of buffer and 0.25 ml of cells. The tubes were shaken and incubated for 50 minutes at room temperature.

The end point was the highest dilution of virus which showed complete hemagglutination. The titer, expressed as hemagglutination units, was the reciprocal of the highest dilution of virus, before the addition of buffer and cells.

Hemagglutination-Inhibition (HI) Test

Alpha Procedure:

This test was prepared in the same manner as the HA test except that 0.25 ml of serum, diluted 1:5, was substituted for 0.25 ml of buffer. The control tube contained 0.5 ml of serum and 0.25 ml of cells.

The serum titer was the reciprocal of the lowest dilution of virus in which hemagglutination was completely inhibited.

The HI titer of the serum was computed from the following formula:

$$\frac{\text{Virus titer}}{\text{Serum titer}} \times \text{dilution of serum} = \text{HI titer}$$

Beta Procedure:

Constant amounts of virus, 10 HA units, were employed with decreasing concentration of serum ranging from 1:5 through 1:2560. The proportion of reagents were the same as used in the Alpha procedure.

The serum titer was the reciprocal of the highest dilution of the serum in which hemagglutination was completely inhibited. The HI titer of the serum was computed from the following formula:

Serum titer X number of HA units = HI titer

Sera

All donors were reared in isolation quarters, and sera were assayed for antibody by the serum neutralization test. Various treatments of sera were performed before dilution to be used in the HI test.

For the production of anti-IB serum, mature roosters were inoculated intratracheally with 0.2 ml of virus infected allantoic fluid, and the serum was collected after six weeks. In one experiment, untreated, trypsin modified, or heat inactivated IBV, was inoculated intravenously or intratracheally.

Viral Infectivity

For determination of viral infectivity, serial ten fold dilutions of the virus were prepared in nutrient broth, or Earle's solution, using separate pipettes per dilution. Five eggs were employed per dilution, and each was inoculated with 0.1 ml via the allantoic cavity. The eggs were incubated at 99 - 99.5 F for five days following inoculation. Embryo mortality during the first 18 hours was considered to be due to non-specific causes, and these were not included in the final results.

Mortality, curling and dwarfing, and urate deposits in the mesonephros were the criteria of infectivity and were used in computing the titer, which was expressed as the

50 percent infectivity end point, ID_{50}, according to the method of Reed and Muench[93].

Serum Neutralization Tests

Dilutions of viruses were prepared in the same manner as described for infectivity titrations. Two rows of tubes were set up parallel to the dilution tubes. One row contained 0.3 ml of serum per tube. The second row contained 0.3 ml of nutrient broth per tube. To each was added 0.3 ml of the corresponding virus dilution. The ingredients were incubated at room temperature for 30 minutes. Serum mixtures were inoculated prior to the control to exclude the possibility of thermal environmental effects on the virus. Inoculation and incubation of eggs were the same as used in viral infectivity titrations.

The 50 percent end point was used to evaluate all titrations. The ID_{50} neutralization index ($ID_{50}NI$) was the difference between the reciprocal of the virus and the serum titers.

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RESULTS

Using Corbo's procedure which gave reproducable results, the time of appearance of HA activity following trypsinization of IBV was studied. This procedure consisted of adding one volume of one percent trypsin to two volumes of IBV infected allantoic fluid. The mixture was incubated at 37 C and samples were removed at various intervals. To this mixture was added one ml of one percent eggwhite trypsin inhibitor. The material was then ready for serial dilution.

The HA activity of IBV 40-23 was first detected after five minutes incubation, whereas with IBV 18-11, activity appeared in 15 minutes. This was the first sampling period for this virus. With both viruses the titer was 80, and progressed to a maximum titer of 10,240 and 640 at the respective sampling time (Table I and II; Figure 1).

It was immediately apparent that the incubation time proposed for this technique was inconvenient. Projects were proposed involving series of HA determinations which should be completed in one day. Trypsinized IBV infected allantoic fluid could not be stored at -30 C or 4 C with any degree of stability. Attempts were made to shorten the incubation period without loss of accuracy.

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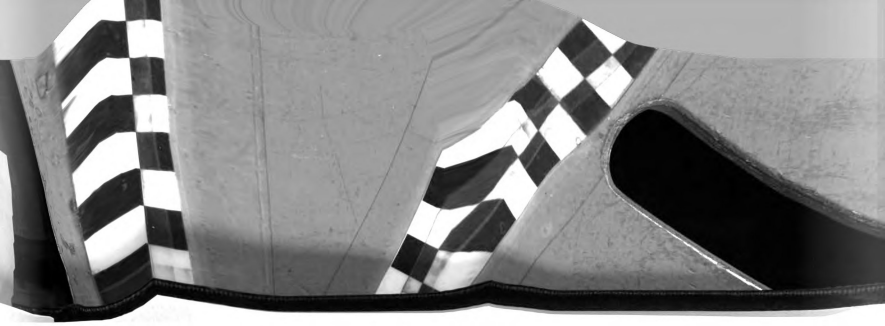


TABLE I

THE HEMAGGLUTINATION TITER OF IBV 18-11
MODIFIED BY TRYPSIN AT 37 C

Time (Minutes)	HA Titer
0	0
15	80
30	160
45	320
60	320
90	320
120	640
150	640
180	640

TABLE II

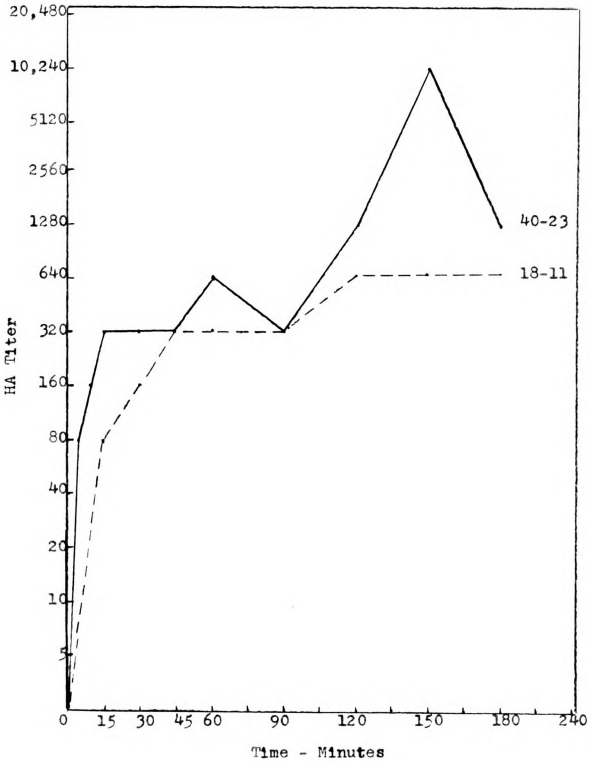
THE HEMAGGLUTINATION TITER OF IBV 40-23
MODIFIED BY TRYPSIN AT 37 C

Time (Minutes)	HA Titer
0	0
5	80
10	160
15	320
30	320
45	320
60	640
90	320
120	1280
150	10,240
180	1280
240	2560

20,48
10,24
51
25
12
64
320
160
80
40
20
10
5
0
HA Titer

FIGURE 1

THE HEMAGGLUTINATION TITER OF IBV 18-11 AND 40-23 MODIFIED BY TRYPSIN AT 37 C



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Cultures of IBV 17-40, 40-23, and 41-8 were incubated with trypsin at 24, 37, and 56 C; and samples were removed at one-half, one, two, and three hours. The HA titers after one-half hour incubation at 56 C were comparable to those after three hours at 37 C (Table III; Figures 2, 3, and 4). Upon further incubation with trypsin, the HA activity of 17-37 and 40-19 decreases slightly, levels off and remains constant until the termination of the test. The sudden loss of activity of IBV 41-8 is not explainable at this time. It may be a strain difference in HA activity worthy of further study.

The concentration of the filtrate from a one percent aqueous suspension of trypsin is probably not constant. The least amount of trypsin necessary to produce consistant maximal HA activity should be known to reduce extraneous material. A phosphate buffer of pH 8.2, the optimal for trypsin solubility, was prepared to which varying amounts of trypsin were added.

Further studies of possible modifications of Corbo's procedure were made using different concentrations of trypsin and ETI. The HA titers after incubation at 56 C for 30 minutes were compared to those at 37 C for three hours (Table IV).

The results clearly indicate that the only modification of Corbo's procedure which can be made without loss of precision is one of temperature and time. When trypsin

Virus

17-37

40-19

42-8

TABLE III

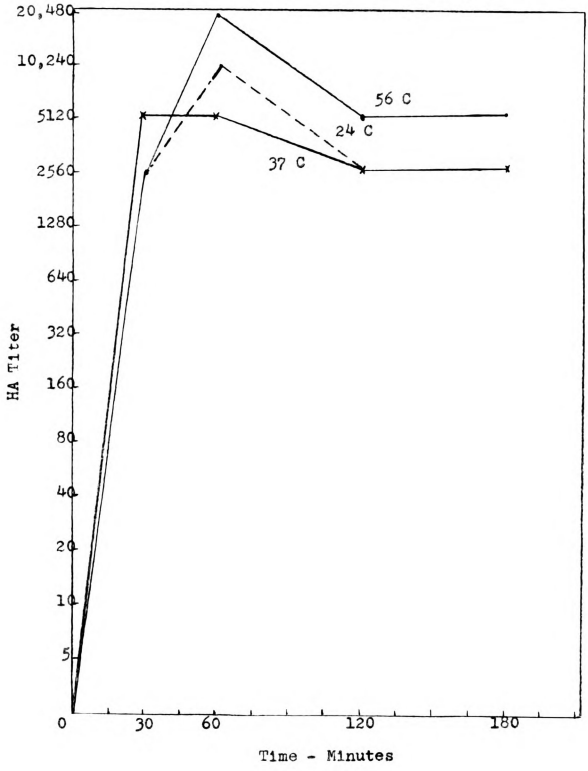
THE EFFECT OF TIME AND TEMPERATURE
ON THE INDUCTION OF HEMAGGLUTINATION
OF IBV 17-37, 40-19, AND 41-8 BY TRYPSIN

Virus	Temperature	HA Titer Time			
		30 Min.	1 Hr.	2 Hrs.	3 Hrs.
17-37	24	2560	10,240	2560	2560
	37	5120	5120	2560	2560
	56	2560	20,480	5120	5120
40-19	24	640	2560	1280	2560
	37	2560	1280	5120	1280
	56	5120	5120	1280	640
41-8	24	10	40	40	0
	37	10	10	80	80
	56	80	0	0	0

FIGURE 2

THE EFFECT OF TIME AND TEMPERATURE

ON THE INDUCTION OF HEMAGGLUTINATION OF IBV 17-37 BY TRYPSIN

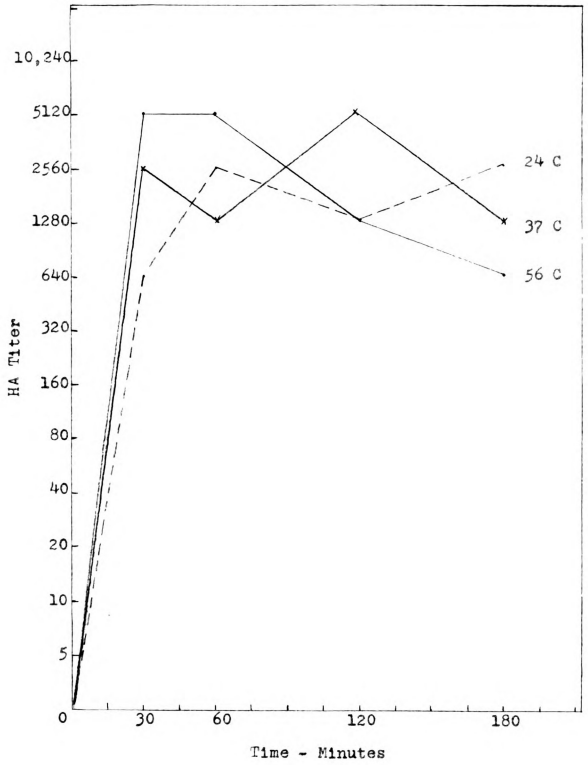


HA Titer

FIGURE 3

THE EFFECT OF TIME AND TEMPERATURE

ON THE INDUCTION OF HEMAGGLUTINATION OF IBV 40-19 BY TRYPSIN



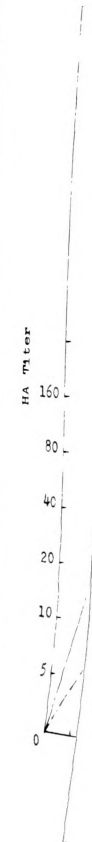

ON THE I
HA Titer
160
80
40
20
10
5
0

FIGURE 4

THE EFFECT OF TIME AND TEMPERATURE
ON THE INDUCTION OF HEMAGGLUTINATION OF IBV 41-8 BY TRYPSIN

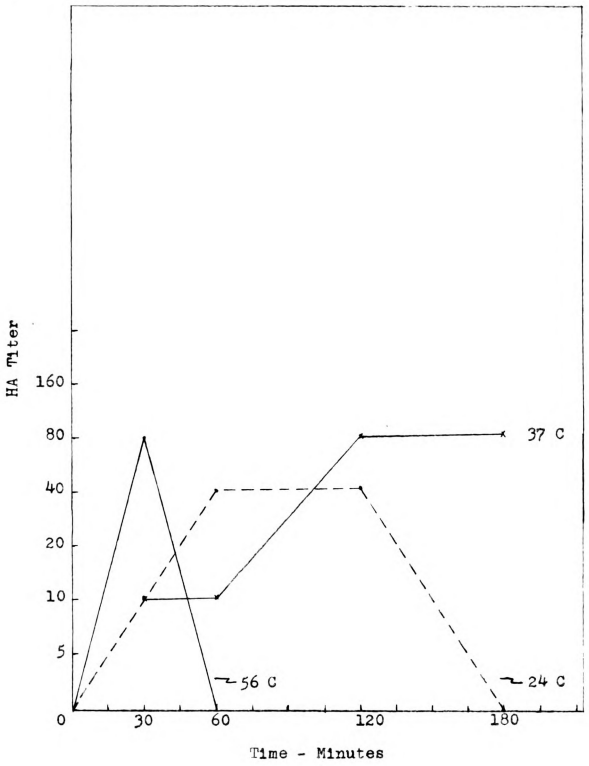


TABLE IV

COMPARISON OF TIME-TEMPERATURE RELATION AND CONCENTRATION OF TRYPSIN-EGGWHITE TRYPSIN INHIBITOR USED FOR THE INDUCTION OF HA OF IBV

Virus	HA Titer					
	37 C for 3 Hrs.	56 C for 30 Min.				
	1% Trypsin 1% ETI	1% T 1% ETI	.1% T .1% ETI	1% T .1% ETI	.1% T 1% ETI	.01% T .01% ETI
17-40	1280	1280	10	160	0	0
40-23	2560	2560	80	640	0	0
41-6	320	640	20	160	0	

and ETI are used in concentrations of less than one percent, the HA titers show marked reduction. Trypsin is necessary for HA activity and the concentration must be greater than 0.01 percent before the activity becomes detectable. The phosphate buffer is not inhibitory to either the trypsin action or the virus reaction with the erythrocytes.

The procedure used in the remainder of this work for the induction of HA activity of IBV is the addition of one volume of a one percent aqueous solution of trypsin to two volumes of virus infected allantoic fluid followed by incubation at 56 C for 30 minutes. Upon removal, one volume of one percent ETI is added, and the mixture is diluted as before.

Although the time of incubation of virus with trypsin can be reduced to 30 minutes, it would be more practical if a quantity of virus could be trypsinized and stored without loss of activity. Studies were performed on treated virus stored at -65 and -30 C and untreated virus at -30 C. The ETI was not added until the treated virus was thawed for use.

Samples at -65 C were stable for three weeks, whereas at -30 C titrations showed reduction of HA activity over the same period. Untreated IBV 40-23 remained stable for three weeks.

The data (Table V) must be considered on a relative basis for each sampling period because it was necessary to

THE

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Time in weeks

1

2

3

4

6

7

TABLE V

THE EFFECT OF TIME AND TEMPERATURE ON THE HA TITER OF TRYPSIN MODIFIED AND UNTREATED IBV 40-23

Time in weeks	HA Titer -65 C Trypsin Modified	-30 C Trypsin Modified	-30 C Untreated
1	1280	2560	1280
2	2560	320	1280
3	1280	640	1280
4	80	40	160
6	2560	320	160
7	640	640	640

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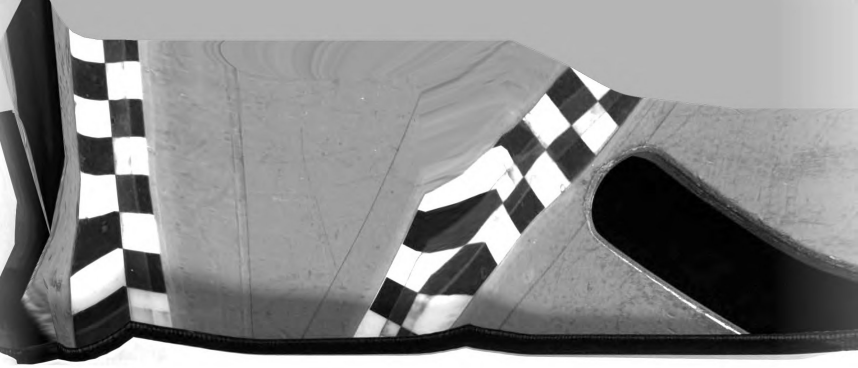
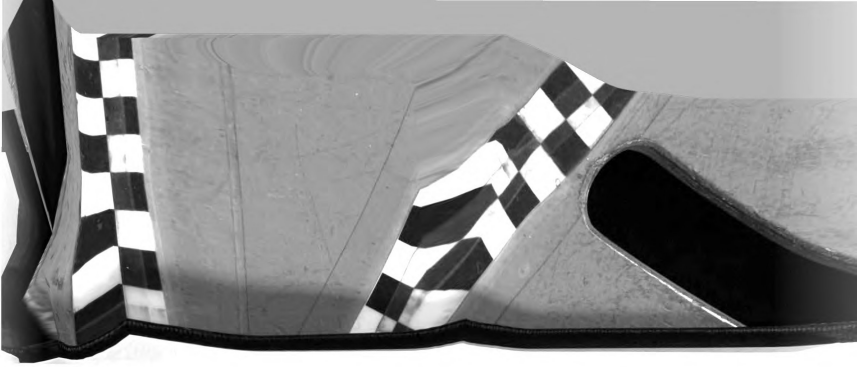


draw fresh cells each week. The discrepancy observed at the fourth and seventh week may be due only to variations in reactivity of cells.

Chicken cells which are collected in Alsever's solution can be used for testing HA activity of IBV. The cells are unsuitable for use after one week of storage at 4 C. Cells colected in saline and stored at 4 C will show reduced HA titers after five days.

Cells from individual chickens vary in their reactivity with the trypsin modified IBV. With some donors, reactivity is decreased after prolonged bleeding schedules.

Investigations were made of the influence of age and of infection of the donor on the reactivity of cells with trypsin modified IBV. Starting with 30 chicks, cells were collected when the chicks were one-day and one-week old. After the cells were collected at one week, the chicks were divided into two groups of 10 each. One group received 0.1 ml of IBV 41-8 intratracheally. The remainder was maintained in a separate room as a control group. For the pre-infection and post-infection periods, it was necessary to collect and pool the blood from several chicks in order to have a sufficient quantity of cells for the HA test. Cells were also collected when the chicks were two, three, four, five, and seven weeks old, or one, two, three, four, and six weeks, respectively, after infection. Hemagglutination was not demonstrable with cells from chicks two weeks old



and only a minimal reaction was detected when they were four weeks old. At the fifth and seventh week, significant HA titers were obtained but there was no clear differential evidence of the influence of cells from infected or control chicks (Table VI; Figure 5).

To test further the influence of age and infection, a similar group of chicks was used. Cells were collected when the chicks were one, eight, and ten days old. On the tenth day, IBV 41-3 was inoculated intratracheally. Cells were also collected at weekly intervals from two to twelve weeks after hatching. No test was performed at the eleventh week.

On the eighth day, and at the 12th week, sera were collected and pooled for respective neutralization tests. The pre-infection $ID_{50}NI$ was $10^{0.34}$. The $ID_{50}NI$ for the control birds was $10^{0.6}$ at the 12th week, whereas the $ID_{50}NI$ for infected birds was $10^{2.5}$. The low NI of the infected birds may be a reflection of either a low antibody level in the decline phase from the maximum which is generally from the sixth to tenth week after infection, or the response of immature birds.

Hemagglutinating activity could not be detected with cells from chicks less than three weeks old. This strain of IBV gave titers of 640 when cells from mature birds were used, but such activity was not demonstrable with the cells from the birds in this experiment.

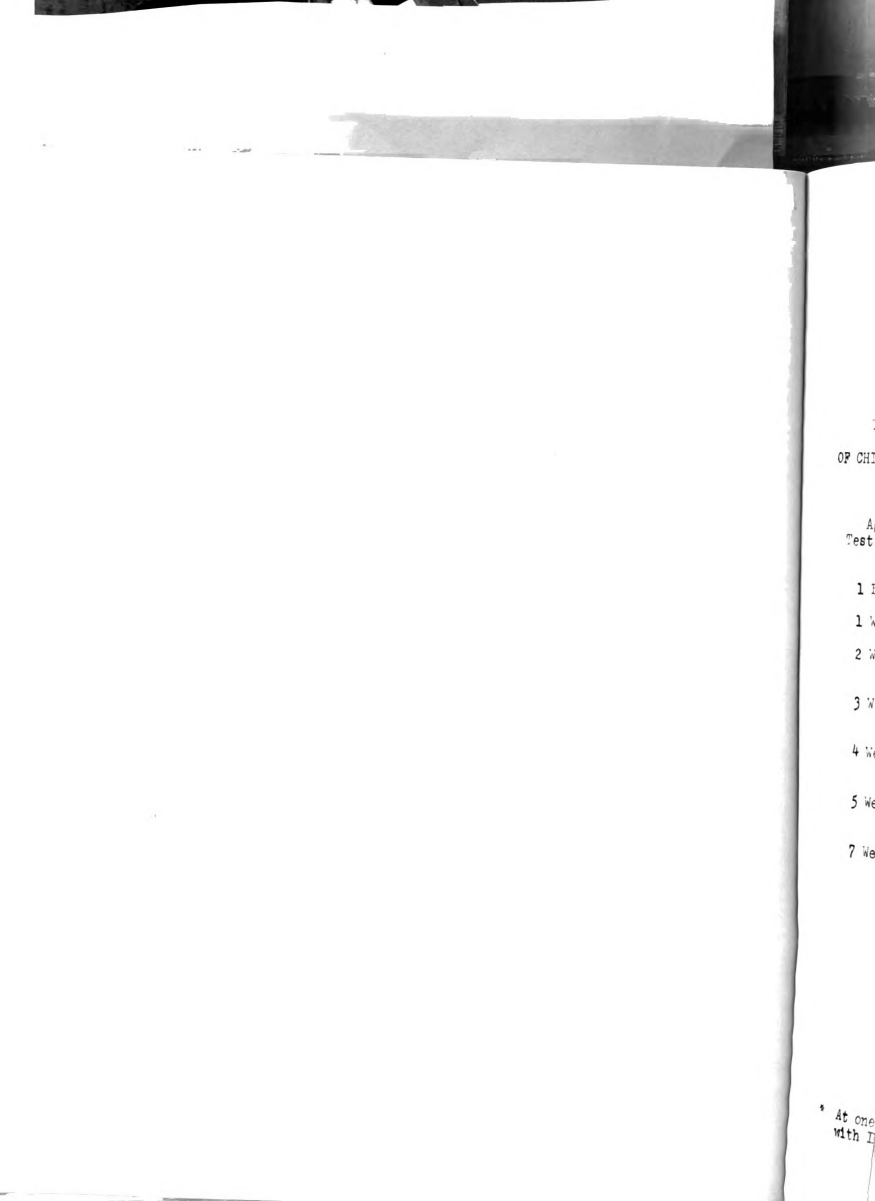
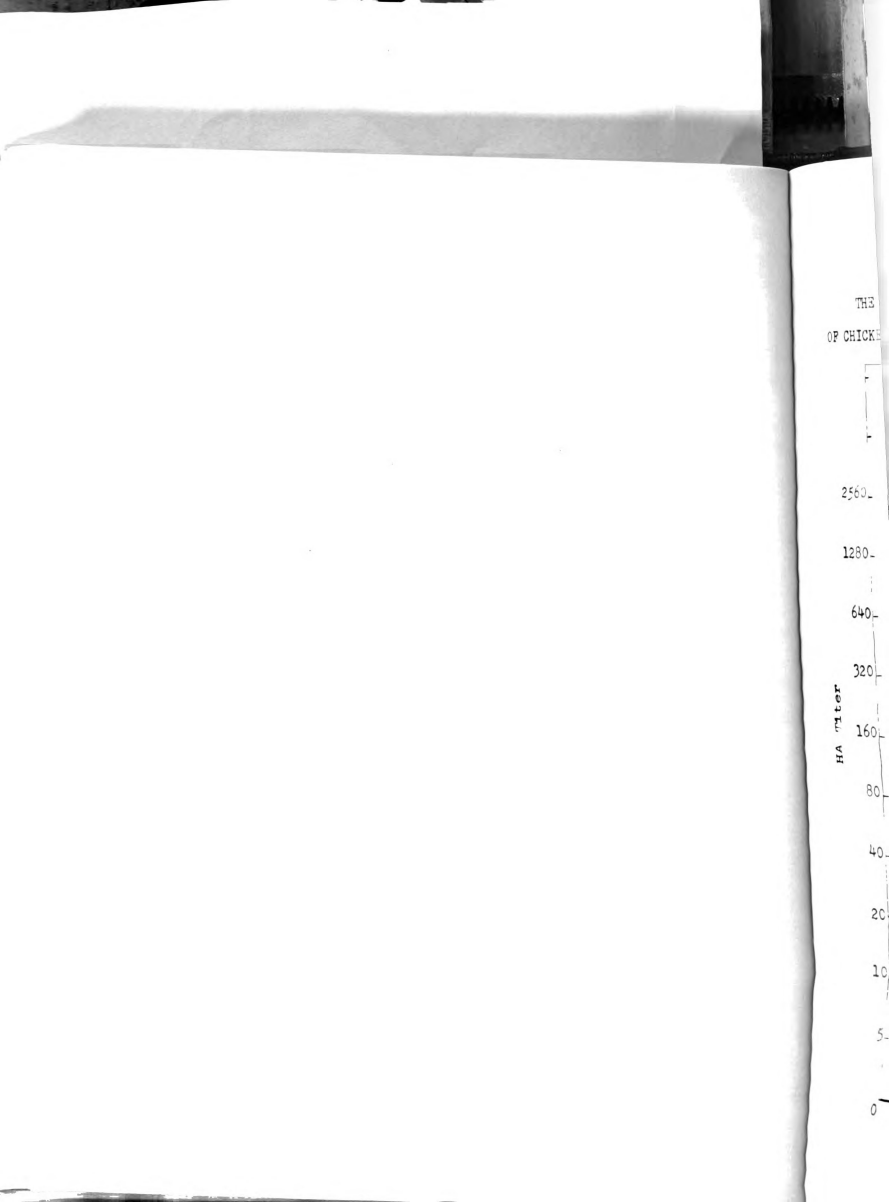


TABLE VI

THE EFFECT OF AGE AND INFECTION ON THE REACTIVITY OF CHICKEN RED BLOOD CELLS WITH THE HEMAGGLUTININ OF IBV 41

Age of Test Bird		Virus for HA	HA Titer
1 Day		41-9	0
1 Week*		41-9	0
2 Week	Infected	41-8	0
	Control		0
3 Week	Infected	41-8	0
	Control		5
4 Week	Infected	41-8	10
	Control		10
5 Week	Infected	41-8	320
	Control		320
7 Week	Infected	41-8	160
	Control		1280

* At one week of age, some of the chickens were infected with IBV Strain 41-9.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20
10
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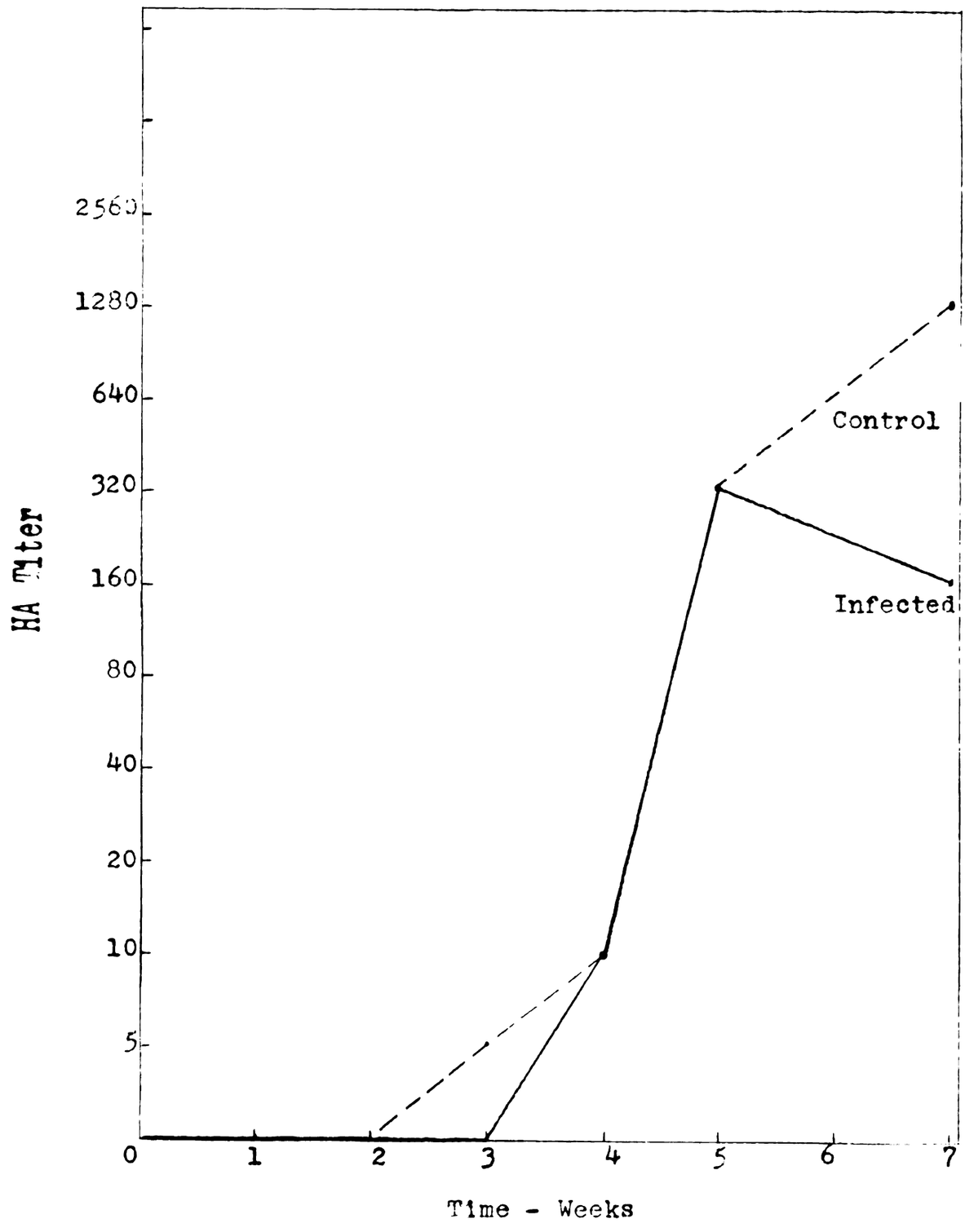
FIGURE 5

THE EFFECT OF AGE AND INFECTION ON THE REACTIVITY OF CHICKEN RED BLOOD CELLS WITH THE HEMAGGLUTININ OF IBV 41



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Fluctuation of HA activity occurred throughout the experiment (Table VII; Figure 6). The HA titers using cells from infected birds paralleled those using cells from the control birds. It would seem most likely that variations in HA activity were due to the cells of this particular group of chicks because IBV 41 was used in both experiments.

Chickens three weeks old or older, poults, and turkeys can be used as donors of cells for the HA test. Cells from the cow, horse, sheep, pig, rabbit, hamster, guinea pig, blue goose, mallard, mute swan, and snow goose could not be used. Cells from 18-day-old chicken embryos were not reactive.

The thermostability of the hemagglutinin of several strains of IBV at 56 C was studied using the trypsinization procedure described by Corbo[25]. Tubes, 12 X 75 mm, containing two ml of virus, were placed in a 56 C water bath. At 30 minute intervals, one tube was removed and one ml of one percent trypsin was added. After re-incubation at 37 C in a water bath for three hours, the tube was removed and one ml of one percent ETI was added. Contrary to Corbo's finding, the HA activity was stable for 180 minutes (Table VIII).

To compare the thermostability of viral infectivity and HA activity, five ml of virus was added to an appropriate number of 13 X 100 mm tubes. At each test interval, one ml of fluid was removed, cooled, and frozen for future

TABLE VII

THE EFFECT OF AGE AND INFECTION ON THE REACTIVITY OF CHICKEN RED BLOOD CELLS WITH THE HEMAGGLUTININ OF IBV 41-3

Age of Test Bird		HA Titer
1 Day		0
8 Days*		
10 Days**		0
2 Weeks	Infected	0
	Control	0
3 Weeks	Infected	5
	Control	5
4 Weeks	Infected	20
	Control	5
5 Weeks	Infected	20
	Control	5
6 Weeks	Infected	0
	Control	0
7 Weeks	Infected	20
	Control	5
8 Weeks	Infected	80
	Control	80
9 Weeks	Infected	0
	Control	20
10 Weeks	Infected	160
	Control	80
12 Weeks***	Infected	80
	Control	20

* Serum Neutralization Index equals $10^{0.3}$

** Some of the birds were infected intratracheally with IBV 41-3

*** Serum Neutralization Index of infected chickens equals $10^{2.5}$
Serum Neutralization Index of control chickens equals $10^{0.6}$

FIGURE 6

THE EFFECT OF AGE AND INFECTION ON THE REACTIVITY OF CHICKEN RED BLOOD CELLS WITH THE HEMAGGLUTININ OF IBV 41-3

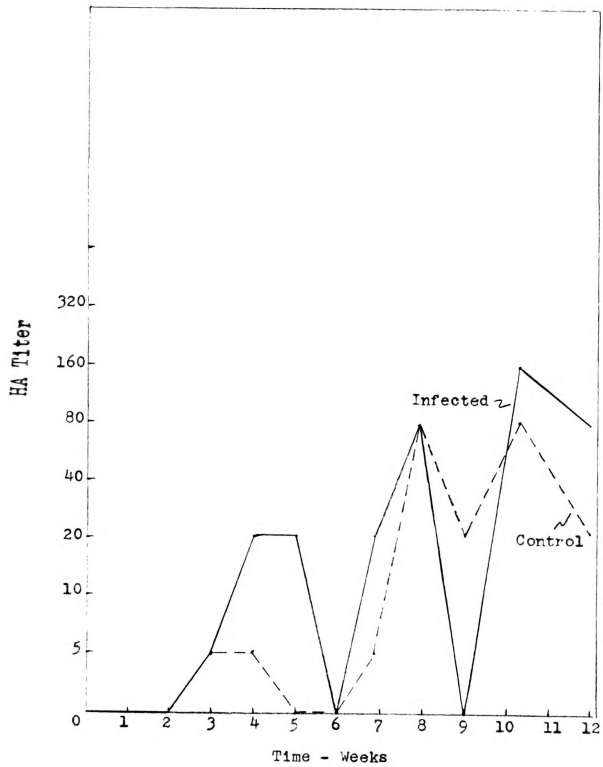

TABLE VIII

THERMOSTABILITY OF THE HEMAGGLUTININ OF TWO DIFFERENT EGG PASSAGES OF IBV 41 AT 56 C

41-7		41-9	
Time (Minutes)	HA Titer (37 C for 3 Hours)	Time (Minutes)	HA Titer (37 C for 3 Hours)
0	1280	0	5120
30	640	30	2560
60	640	60	5120
90	1280		
120	1280	120	5120
150	640		
180	640	180	5120

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infectivity assay. At the same time, two ml was removed for determination of HA activity by the Corbo procedure. The remaining two ml was treated with trypsin at 56 C for 30 minutes. At zero time, one sample was titrated for infectivity, while two others were trypsinized at the two incubation temperatures and respective times. Although the trypsin modified virus was incubated at 56 C for 30 minutes or at 37 C for three hours, the respective titers are recorded for zero time.

The hemagglutinin was thermostable for 180 minutes, whereas the virus was innocuous for chicken embryos within 60 to 90 minutes (Tables IX to XI; Figures 7 to 9).

In an extension of this experiment, IBV 40-23 and 41-9 were incubated at 56 C for nine hours, during which time portions were removed at hourly intervals for HA determination. Trypsinization was performed only at 56 C for 30 minutes, because the thermostability of IBV is independent of the trypsinization procedure. There was no significant decrease of the HA activity after nine hours at 56 C (Table XII and XIII; Figure 10).

To compare the effect of trypsin on the infectivity of IBV 40-23 and 41-7, two samples of each virus, one containing trypsin as used in the HA test and one without trypsin were incubated at 37 C for three hours.

The addition of trypsin to virus resulted in a threefold dilution. To correct for this the log of the

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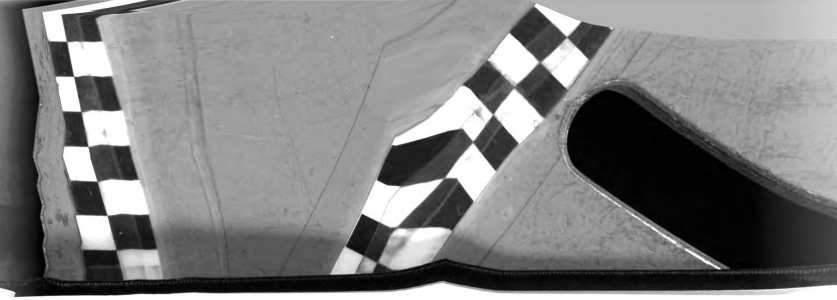

TABLE IX

THERMOSTABILITY OF THE HEMAGGLUTININ AND INFECTIVITY OF IBV 17-37 AT 56 C

Time (Minutes)	HA Titer (37 C for 3 Hours)	HA Titer (56 C for 30 Minutes)	Infectivity Titer
0	1280	1280	7.2
15			3.5
30	640	320	2.2
45			0.8
60	640	320	0.1
90	640	640	0.0
120	640	1280	
180	640	640	

TABLE X

THERMOSTABILITY OF THE HEMAGGLUTININ AND INFECTIVITY OF IBV 16-43 AT 56 C

Time (Minutes)	HA Titer (37 C for 3 Hours)	HA Titer (56 C for 30 Minutes)	Infectivity Titer
0	2560	2560	5.5
15			0.8
30	2560	2560	0.8
45			0.1
60	5120	2560	0.0
120	1280	1280	
180	5120	5120	

TABLE XI

THERMOSTABILITY OF THE HEMAGGLUTININ AND INFECTIVITY OF IBV 3-15 AT 56 C

Time (Minutes)	HA Titer (37 C for 3 Hours)	HA Titer (56 C for 30 Minutes)	Infectivity Titer
0	2560	2560	6.2
15			3.3
30	2560	2560	3.4
45			3.0
60	1280	1280	1.0
75			0.0
120	1280	1280	
180	1280	1280	

TABLE XII

THERMOSTABILITY OF THE HEMAGGLUTININ AND INFECTIVITY OF IBV 40-23 AT 56 C

Time (Minutes)	HA Titer (56 C for 30 Minutes)	Infectivity Titer
0	2560	6.4
15		4.3
30		3.2
45		2.0
60	5120	0.8
75		0.1
90		0.0
120	5120	
150	1280	
180	5120	
210	2560	
240	640	
270	640	
300	640	
330	640	
390	640	

Time
(Hour

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TABLE XIII

THERMOSTABILITY OF THE HEMAGGLUTININ

OF IBV 40-23 AND 41-9 AT 56 C

Time (Hours)	HA Titers (56 C for 30 Minutes)	
	40-23	41-9
0	640	160
3	320	80
4	320	80
5	640	320
6	320	160
7	640	160
8	640	160
9	320	160

FIGURE 7

THERMOSTABILITY OF THE HEMAGGLUTININ AND INFECTIVITY OF IBV 17-37 AT 56 C

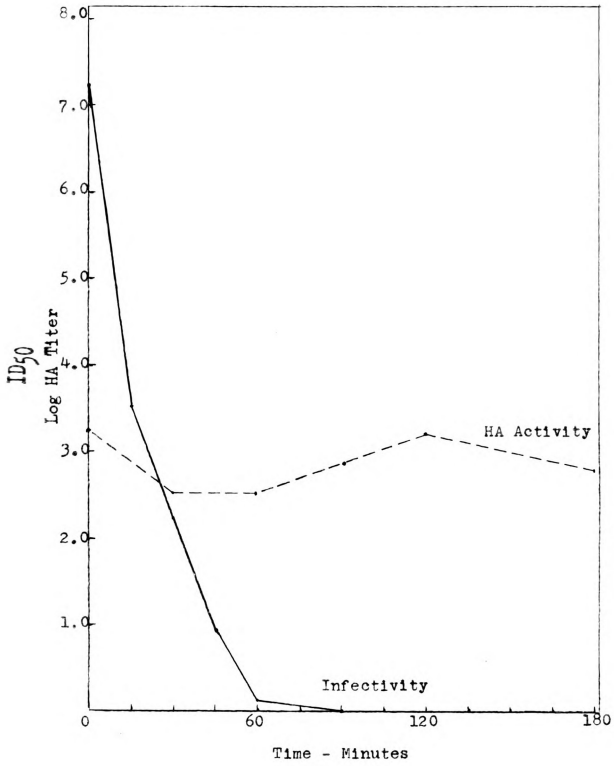


FIGURE 8

THERMOSTABILITY OF THE HEMAGGLUTININ AND INFECTIVITY OF IBV 16-43 AT 56 C

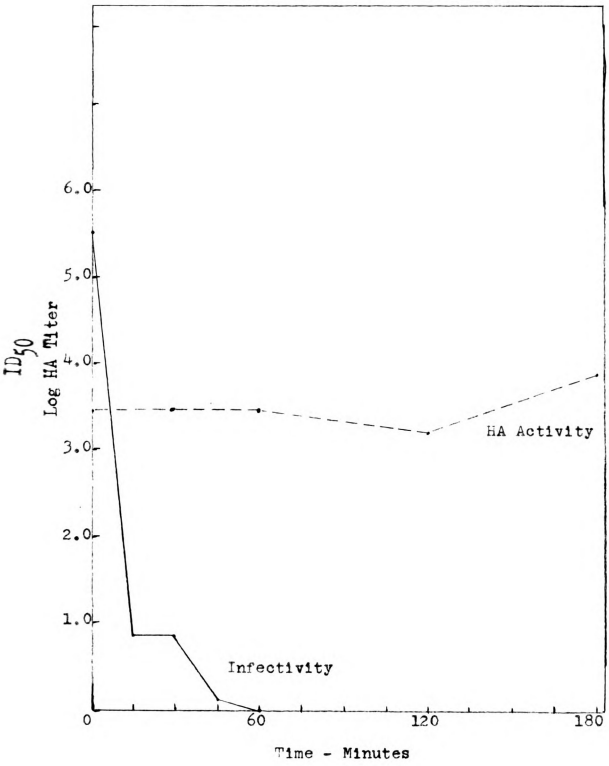


FIGURE 9

THERMOSTABILITY OF THE HEMAGGLUTININ AND INFECTIVITY OF IBV 3-15 AT 56 C

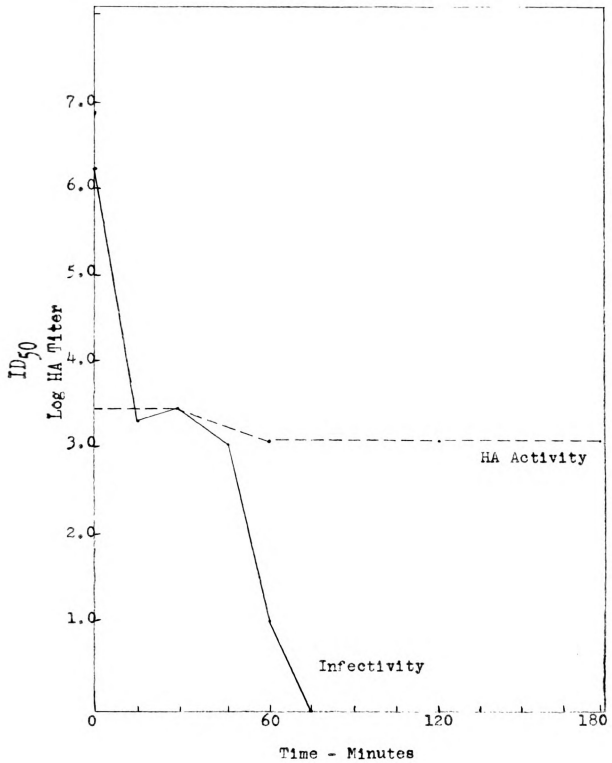
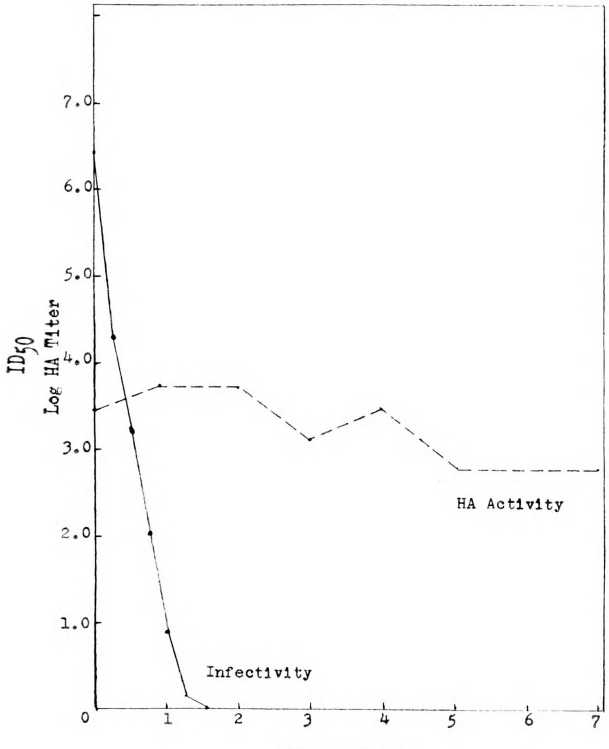


FIGURE 10

THERMOSTABILITY OF THE HEMAGGLUTININ AND INFECTIVITY OF IBV 40-23 AT 56 C



dilution factor (3), $10^{0.5}$, was added to the ID_{50} values.

The reduction of titer of the control samples due to heat inactivation alone must also be taken into consideration and added to the ID_{50} obtained with the trypsinized virus. The corrected values are considered to be a more accurate reflection of any change due to the action of trypsin alone. After three hours, there was a $10^{0.9}$ reduction in viral infectivity of the trypsinized sample as compared to the control. With IBV 40-23, the titer of the control was $10^{0.3}$ lower than that of the trypsinized sample at the same period. The initial titer of the control was $10^{0.6}$ lower than the corrected value of the trypsinized sample. Whether this is due to the disruption of viral aggregates or an error in technique is unknown. Comparison of uncorrected values shows the titer of the control sample to be $10^{0.9}$ lower than the titer of the trypsin treated sample (Table XIV and XV; Figure 11 and 12).

As an extension of this experiment, IBV 3-16 was treated in a similar manner except that the testing period was increased to 20 hours. After three hours there was a reduction of $10^{1.3}$ in the titer of the trypsinized sample. After eight hours, the titers of the trypsinized sample and the control sample were approximately the same. The virus was non-infectious at the 20th hour (Table XVI; Figure 13).

A second sample of IBV 3-16 was tested. After four

TABLE XIV

THE EFFECT OF TRYPSIN AT 37 C ON THE INFECTIVITY OF IBV 41-7

Time (Hours)	Infectivity Titer			
	Trypsin Modified	Correction Value for Dilution	Correction Value for Heat	Control
0	6.6	7.1		7.0
1	6.6	7.1		6.2
2	6.2	6.7	7.0	6.7
3	5.3	5.8	5.8	6.7

TABLE XV

THE EFFECT OF TRYPSIN AT 37 C ON THE INFECTIVITY OF IBV 40-23

Time (Hours)	Infectivity Titer			
	Trypsin Modified	Correction Value for Dilution	Correction Value for Heat	Control
0.0	6.8	7.3		6.7
1.0	5.4	5.9	6.1	
1.5				6.4
2.0	4.6	5.0	5.2	
3.0	4.5	4.9	5.7	5.4

TABLE XVI

THE EFFECT OF TRYPSIN AT 37 C ON THE INFECTIVITY OF IBV 3-16

Time (Hours)	Infectivity Titer			
	Trypsin Modified	Correction Value for Dilution	Correction Value for Heat	Control
0.0	7.6	8.1		8.4
1.0	7.3	7.8	7.8	
1.5				8.2
2.0	6.8	7.3	7.4	
3.0	5.7	6.2	6.5	7.8
8.0	4.3	4.8	6.1	6.5
20.0	0.0			0.0

FIGURE 11

THE EFFECT OF TRYPSIN AT 37 C ON THE INFECTIVITY OF IPV 41-7

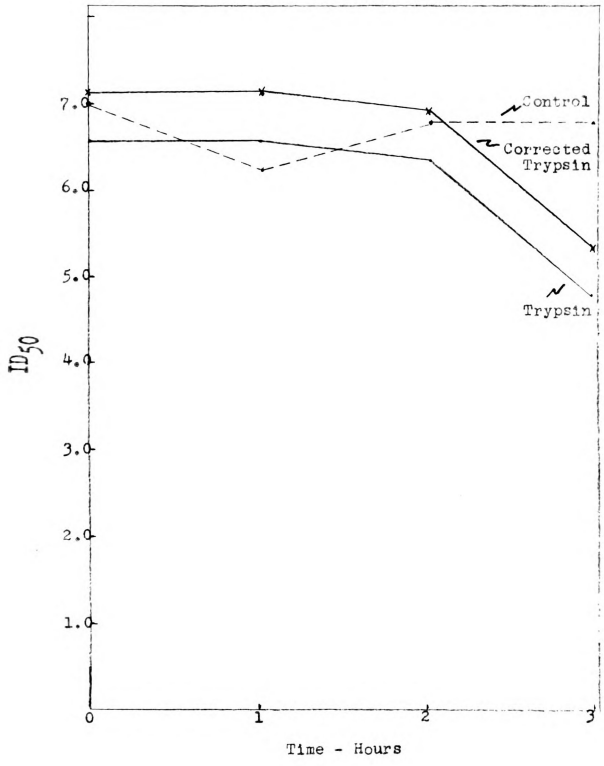


FIGURE 12

THE EFFECT OF TRYPSIN AT 37 C ON THE INFECTIVITY OF IBV 40-23

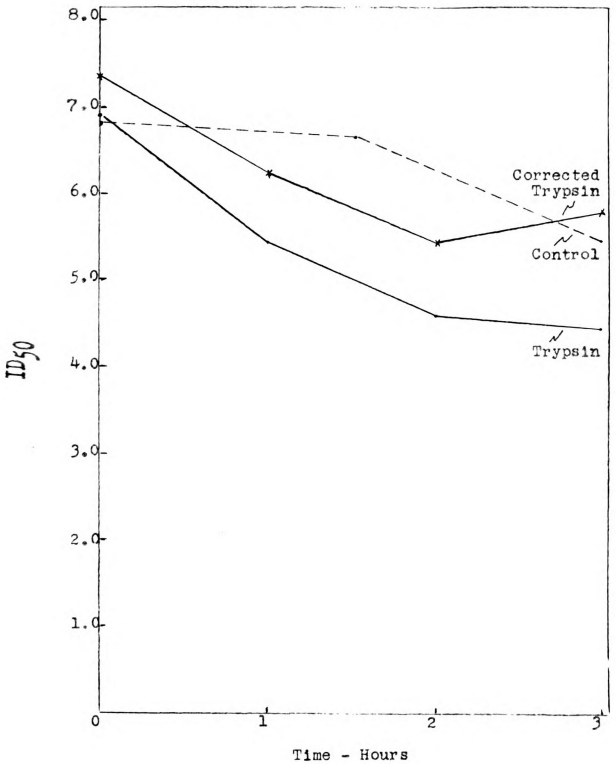
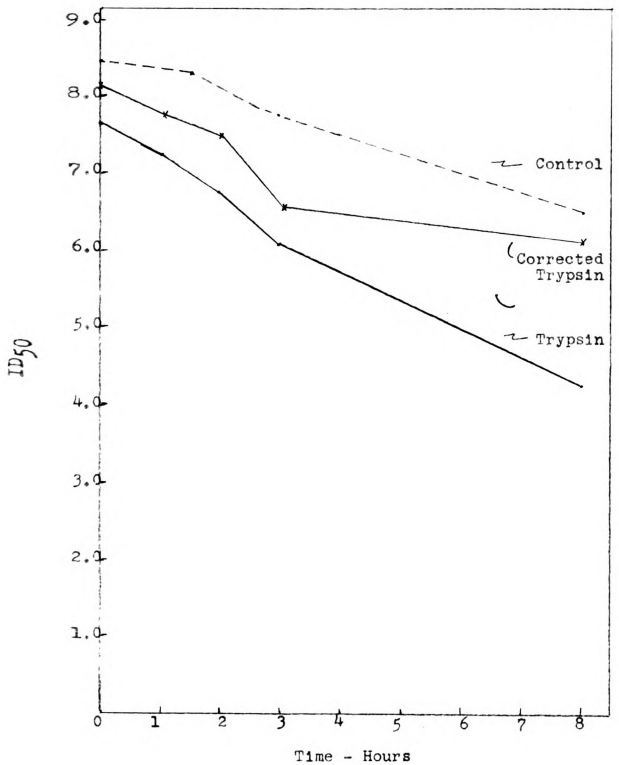


FIGURE 13

THE EFFECT OF TRYPSIN AT 37 C ON THE INFECTIVITY OF IBV 3-16



hours, the titer of the trypsinized sample was $10^{0.6}$ lower than the control. Again the initial titer of the control was $10^{0.7}$ lower than the corrected value of the trypsinized sample. Analysis of uncorrected values reveals that the titer of the trypsin treated sample was $10^{1.5}$ lower than the control. At the eighth and 15th hour, the corrected titers of both samples were equal. Infectivity was observed after 23 hours of incubation, but an end point was not determined (Table XVII; Figure 14).

IBV 41-9 was tested at zero, 12, 16, and 20 hours. Infectivity was observed at the 20th hour with the titer of the trypsinized sample being greater than that of the control (Table XVIII).

To determine the correlation between viral multiplication as detected by infectivity and HA activity following inoculation of embryonating chicken eggs, 60 eggs were inoculated, respectively, with 0.1 ml of undiluted and a 10^{-3} dilution of IBV 41-6. Immediately after inoculation and at four, eight, and 12 hours, and at subsequent 12 hour intervals through 72 hours, five eggs containing living embryos were selected at random and chilled for 12 hours at 4 C. The allantoic fluid from these embryos was then collected and pooled, and infectivity and HA titrations were performed. The remaining fluid was stored at -30 C for tests to be done one week later to determine the effect of freezing and

TABLE XVII

THE EFFECT OF TRYPSIN AT 37 C ON THE INFECTIVITY OF IBV 3-16

Time (Hours)	Infectivity Titer			
	Trypsin Modified	Correction Value for Dilution	Correction Value for Heat	Control
0	7.7	8.2		7.5
2	7.2	7.7	7.8	7.4
4	5.5	6.0	6.4	7.0
8	4.8	5.3	6.1	6.2
12	4.5	5.0	6.7	4.4
15	3.2	3.7	4.1	4.0
23	>0.0			>0.0

TABLE XVIII

THE EFFECT OF TRYPSIN AT 37 C ON THE INFECTIVITY OF IBV 41-9

Time (Hours)	Infectivity Titer		
	Trypsin Modified	Correction Value for Dilution	Control
0	8.3	8.8	8.8
12	4.2	4.7	>6.0
16	2.8	3.3	>6.0
20	3.0	3.5	1.8

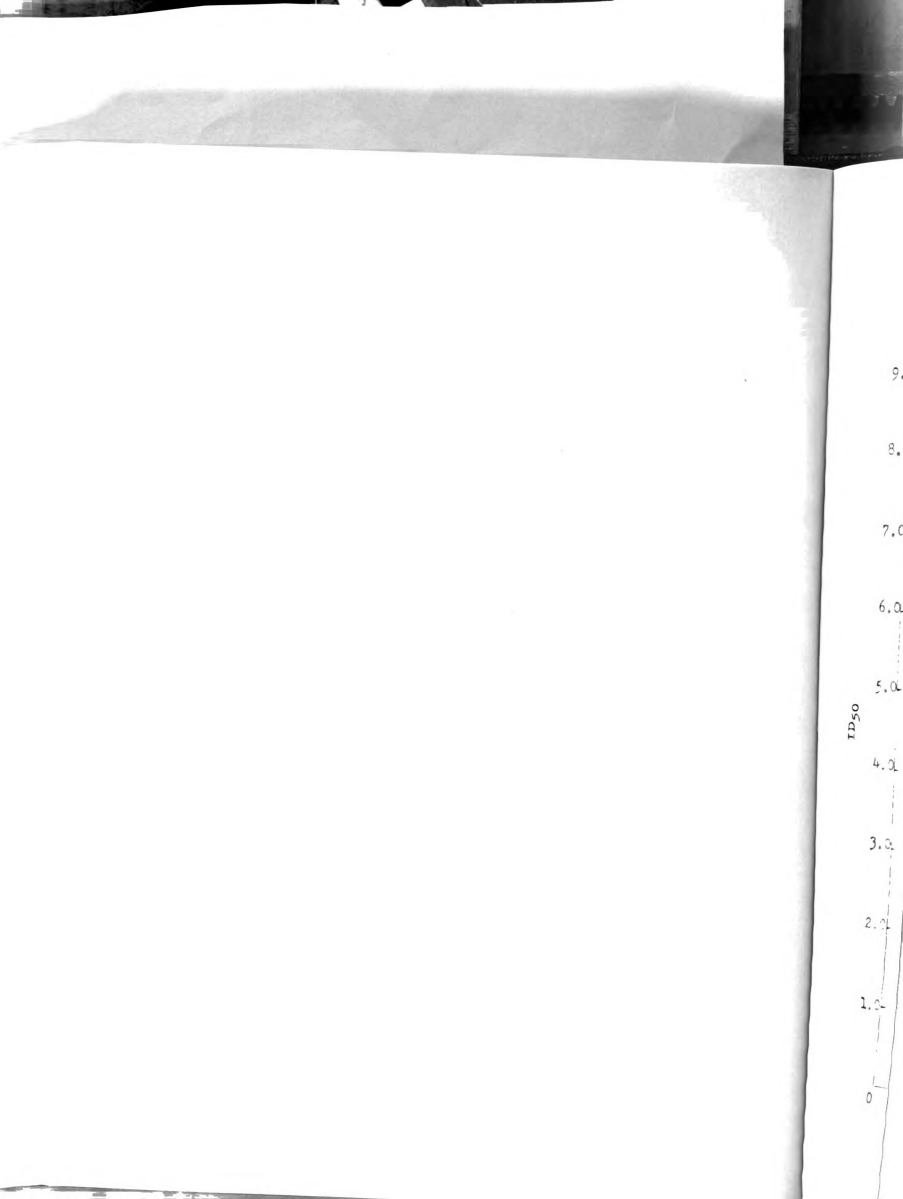
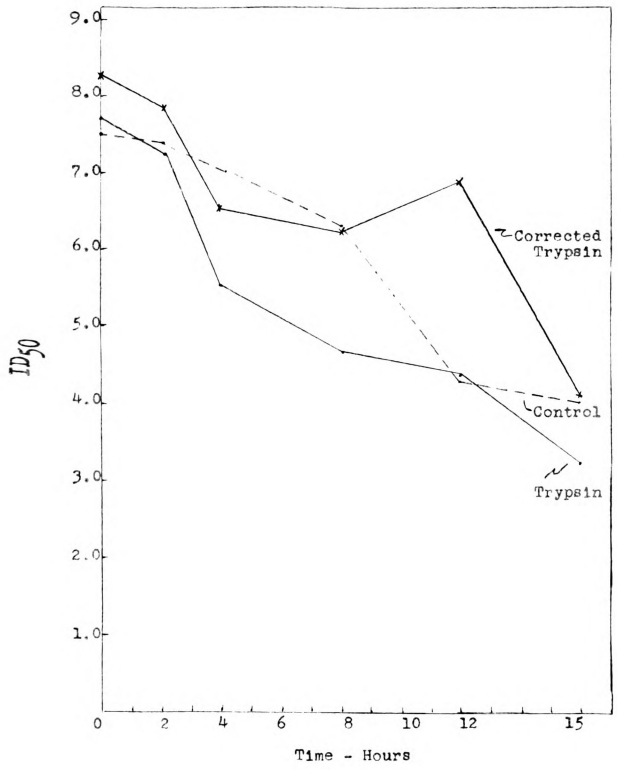

9.
8.
7.0
6.0
5.0
ID50
4.0
3.0
2.0
1.0
0

FIGURE 14

THE EFFECT OF TRYPSIN AT 37 C ON INFECTIVITY OF IBV 3-16



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thawing on HA activity of the fluid as compared to the fluid which had not been frozen.

Infectivity titrations of IBV 41-6 showed the typical lag, log, decline and stationary phases of viral multiplication (Table XIX and XX; Figure 15 and 16).

With the undiluted inoculum, the ID_{50}, $10^{4.0}$, at the fourth hour, represented a $10^{2.4}$ decrease from the ID_{50} $10^{6.4}$ of the inoculum. The log phase reached a maximum of ID_{50} $10^{8.9}$ at the 24th hour, or a $10^{4.9}$ increase in infectivity. Following this, there was a $10^{2.2}$ decline to $10^{6.7}$ at the 48th hour followed by the stationary phase through the 72nd hour.

Hemagglutinating activity of the frozen sample could first be detected at zero time when the titer was 10. There was a progressive increase to a titer of 640 at the 24th hour. With the exception of the titer of 320 obtained at the 48th hour, the titer continued to increase to 1280 at the 72nd hour.

The ID_{50} of the 10^{-3} diluted inoculum of IBV 41-6 was $10^{1.2}$ at the fourth hour. The maximum ID_{50}, $10^{7.8}$, was reached at the 24th hour, or an increase of $10^{6.6}$. This was followed by minor fluctuations in a general decrease to $10^{7.4}$ at the 72nd hour.

Only with the frozen sample could hemagglutinating activity be detected four hours after inoculation when the titer was 10. The titer remained constant at 80 from the

TABLE XIX

INFECTIVITY AND HEMAGGLUTINATING ACTIVITY

OF IBV 41-6

Undiluted Inoculum

Time (Hours)	Infectivity Titer	HA Titer	
		Before Freezing	After Freezing
Original Titer	6.4		320
0	5.0	40	10
4	4.0	10	40
8	6.4	20	40
12	8.0	160	320
24	8.9	160	640
36	7.8	640	640
48	6.7	2560	320
60	6.8	2560	1280
72	6.8	640	1280

TABLE XX

INFECTIVITY AND HEMAGGLUTINATING ACTIVITY

OF IBV 41-6

Inoculum Diluted 10^{-3}

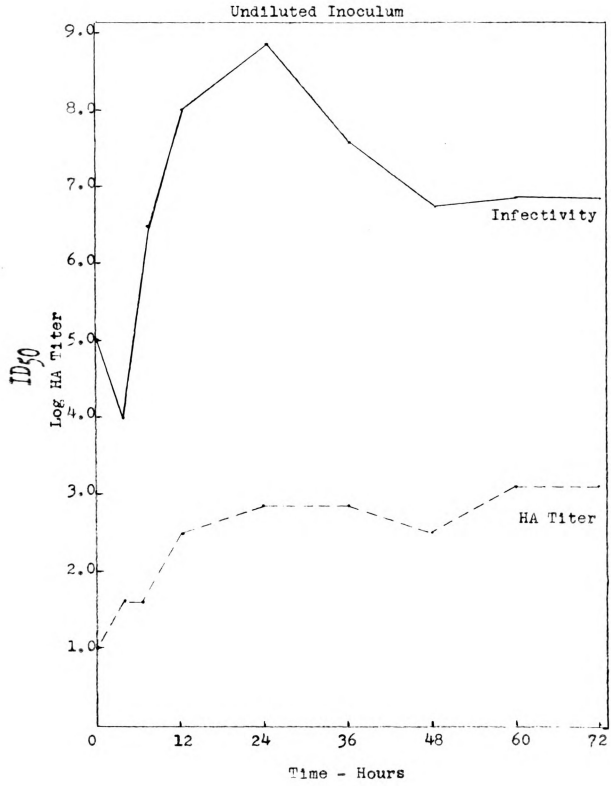
Time (Hours)	Infectivity Titer	HA Titer	
		Before Freezing	After Freezing
Original Titer	6.6		0
4	1.2	0	10
8	4.1	160	80
12	6.8	160	80
24	7.8	80	40
36	7.3	320	80
48	7.6	320	320
60	6.8	2560	640
72	7.4	320	1280

6.
ID_{50}
log HA titer
3.
2.
1.
0

FIGURE 15

INFECTIVITY AND HEMAGGLUTINATING ACTIVITY

OF IBV 41-6



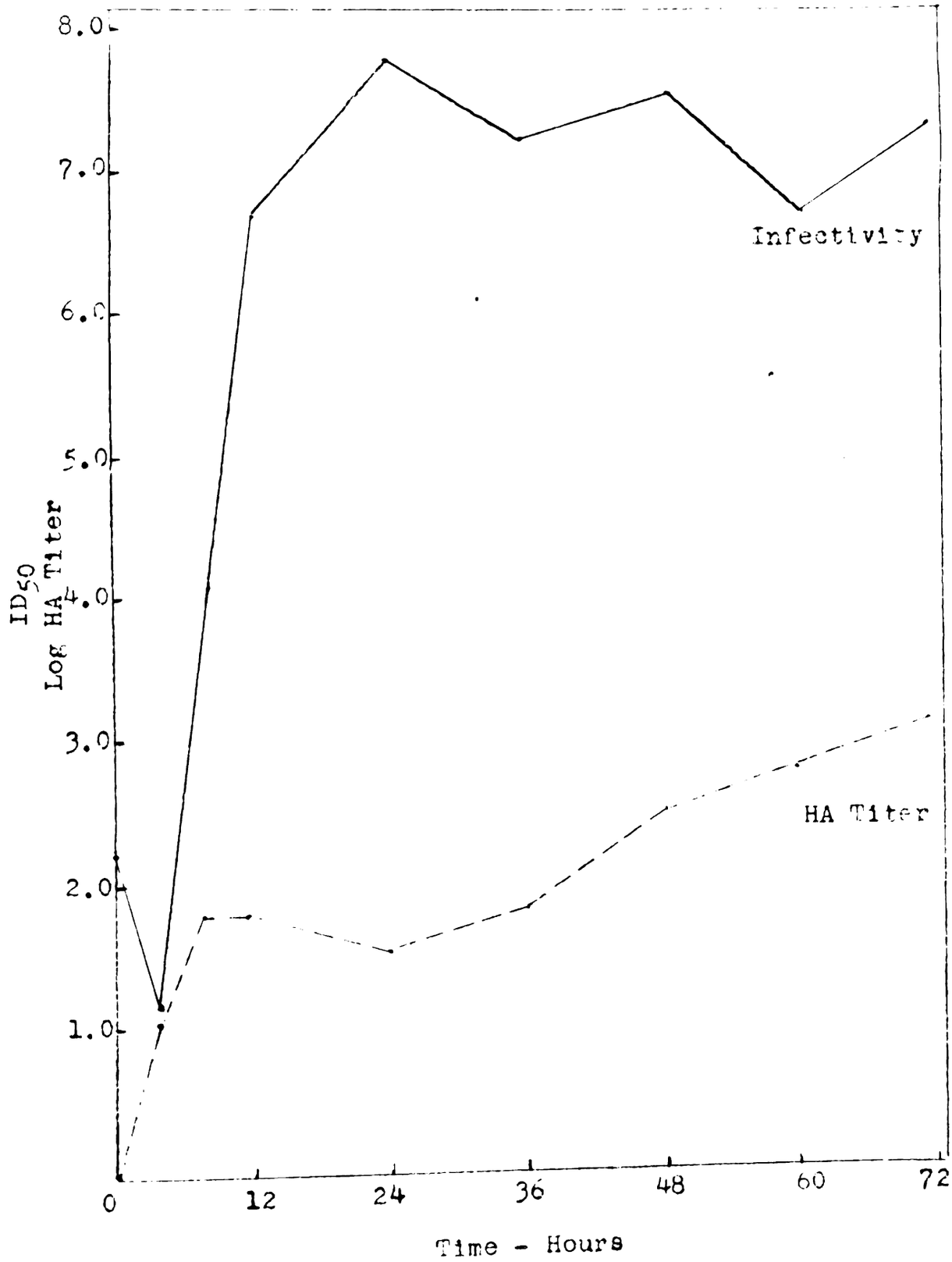
ID_{50}
Log HA Titer

FIGURE 16

INFECTIVITY AND HEMAGGLUTINATING ACTIVITY

OF IBV 41-6

Inoculum Diluted 10^{-3}



8th through the 36th hour and then increased to 1280 at the 72nd hour. This general trend in HA activity was paralleled rather closely with the unfrozen samples.

Using IBV 40-23 and 17-40 with the same procedure as previously described, infectivity tests were performed with unfrozen allantoic fluid whereas HA activity was determined only with samples frozen for one week. The test periods ranged from 12 through 101 hours after inoculation (Table XXI and XXII; Figure 17 and 18).

The maximum infectivity titer of IBV 40-23 was $10^{8.3}$ at the 24th hour, which represented a $10^{2.1}$ increase from the original inoculum of $10^{6.4}$. The titer decreased to 6.3 at the 72nd hour and remained constant until the termination of the experiment. The HA activity was not detected until the 72nd hour when the titer was 160. At the 101st hour, the titer was 1280.

The titer of IBV 17-40 remained at $10^{7.2}$ through the 24th hour, decreased to $10^{6.5}$ at the 48th hour, and then remained rather constant to the 101st hour. HA activity was first detected at the 48th hour with a titer of 160 and to a titer of 2560 at the 72nd hour. At the 101st hour, HA activity was not detected.

With only the frozen samples, the HA activity of all strains reached a maximum at 60 hours or later. The maximum infectivity of all strains was reached at 24 hours except for IBV 17-40. The titer of this virus remained

TABLE XXI

INFECTIVITY AND HEMAGGLUTINATING ACTIVITY

OF IBV 40-23

Undiluted Inoculum

Time (Hours)	Infectivity Titer	HA Titer After Freezing
Original Inoculum	6.2	320
12	6.8	0
24	8.3	0
48	7.2	0
72	6.3	160
101	6.2	1280

TABLE XXII

INFECTIVITY AND HEMAGGLUTINATING ACTIVITY

OF IBV 17-40

Undiluted Inoculum

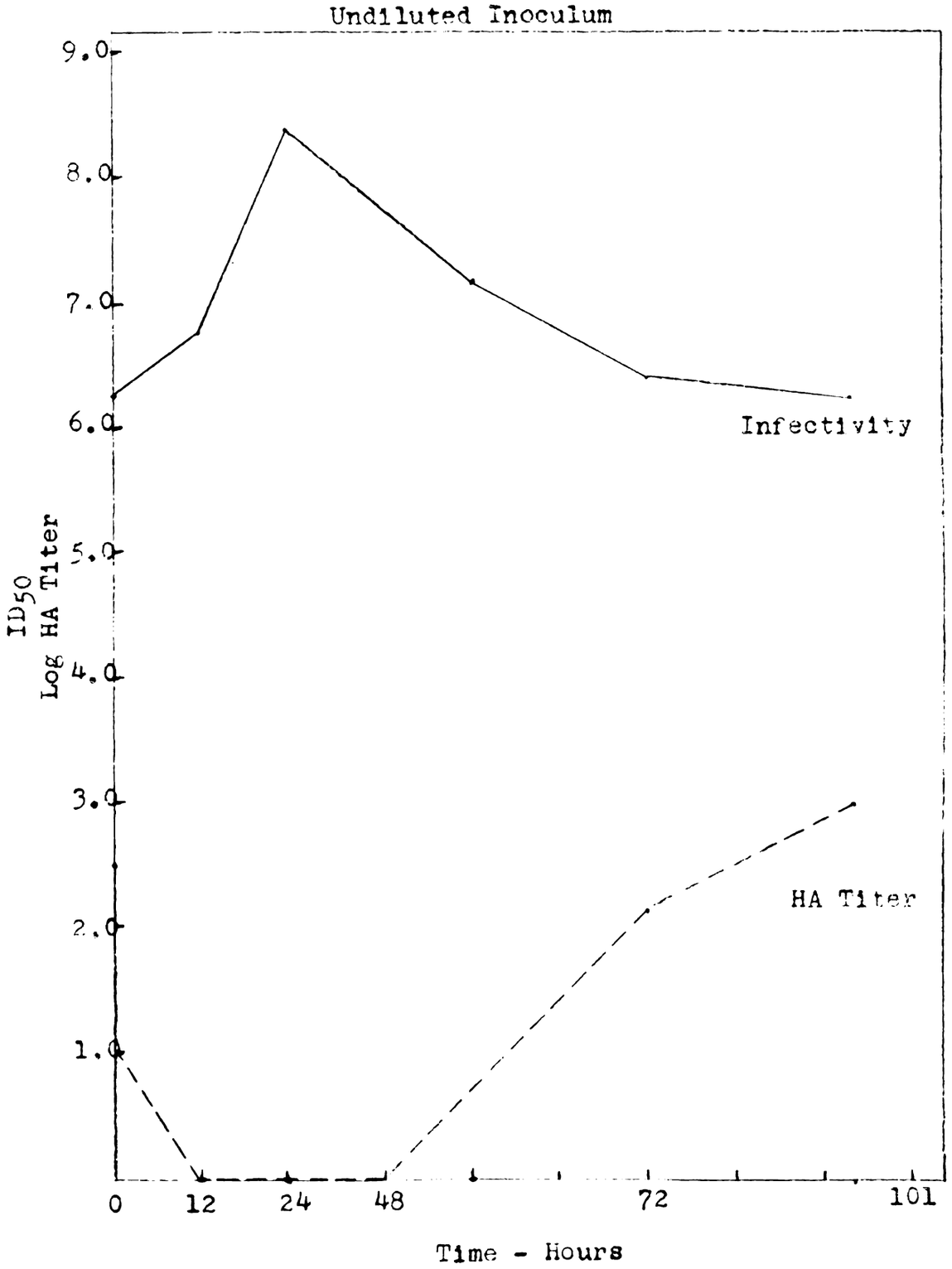
Time (Hours)	Infectivity Titer	HA Titer After Freezing
Original Inoculum	7.2	320
12	7.2	0
24	7.2	0
48	6.5	160
72	6.5	2560
101	6.2	0

ID_{50}
Log HA Titer
3.0
2.0
1.0
0

FIGURE 17

INFECTIVITY AND HEMAGGLUTINATING ACTIVITY

OF IBV 40-23



LD_{50} of HA Titer

3.0

2.0

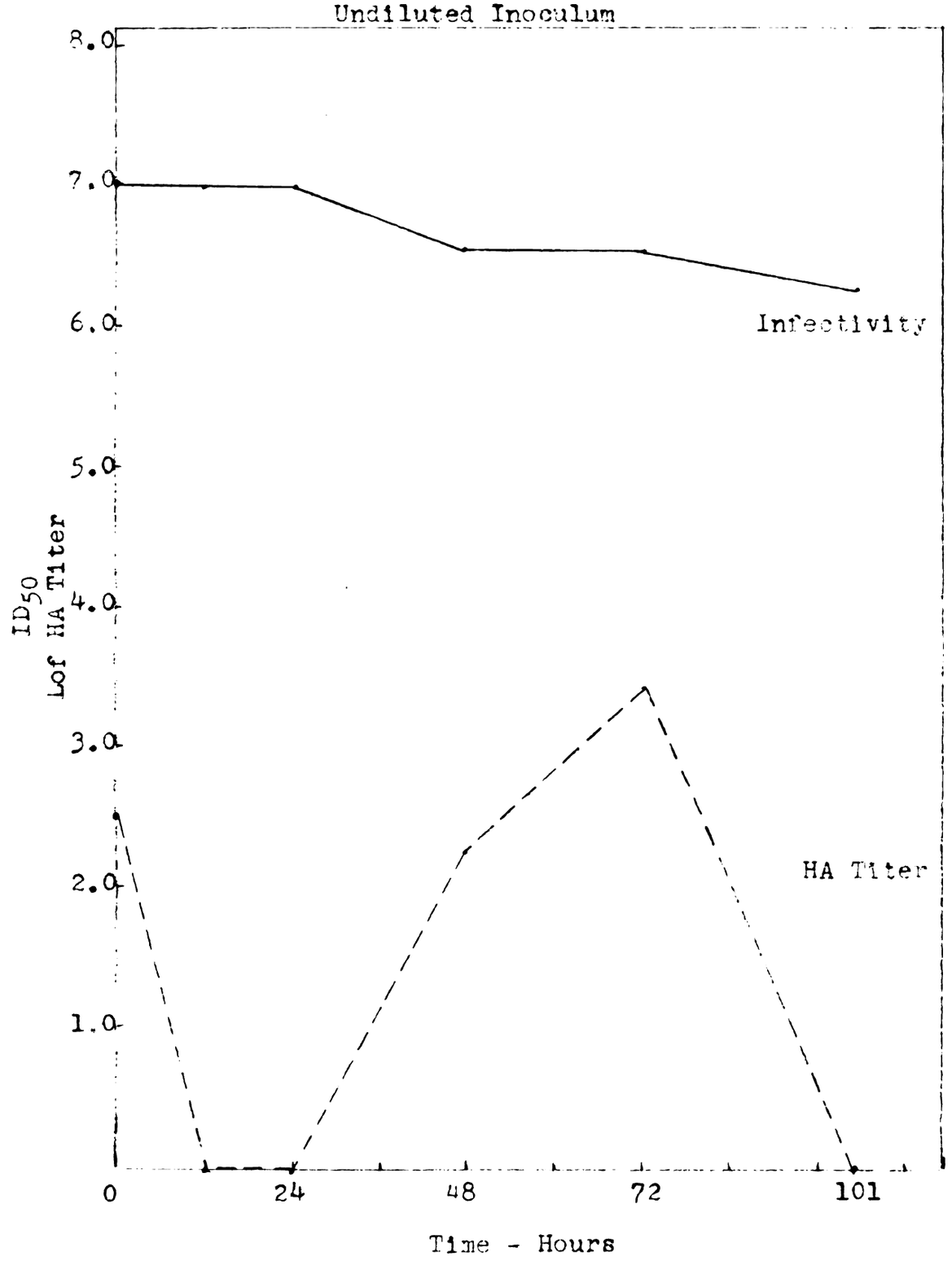
1.0

0

FIGURE 18

INFECTIVITY AND HEMAGGLUTINATING ACTIVITY

OF IBV 17-40



constant through the 24th hour and then decreased. Although there is no evidence of correlation between infectivity and HA activity, practical application can be made of these data. Harvests of allantoic fluid at 24 hours post inoculation yield high infectivity titers but little or no HA activity. To obtain high yields of HA activity, the allantoic fluid should be harvested after 60 hours.

In an attempt to determine the role of the infective viral particle in the HA test, a sample of virus infected allantoic fluid, which had been treated with trypsin at 37 C for three hours, was serially diluted in Earle's solution under sterile conditions. A modification of the standard HA test was used. Instead of the conventional 0.25 ml volume of ingredients, 0.5 ml portions of the appropriate virus dilutions and cell suspensions were mixed in order to have sufficient material for infectivity tests. For the control, 0.5 ml of Earle's solution was substituted for the cell suspension . All tubes were incubated at room temperature for 45 minutes. At the end of this time, hemagglutination was complete in the 1:80 dilution of the virus; and negative in the 1:160 dilution of virus. After centrifugation, the cell free supernatant fluid from the virus dilutions of 1:40, 1:80, and 1:160 were used for titrations of infectivity.

If the infective particle was adsorbed to the cells, it would be expected that a reduction of infectivity of the supernatant fluid would be detected as compared to the

control in which cells were not present. With the 1:40 dilution of the virus, the infectivity titer of both samples was the same. With the 1:80 and the 1:160 dilutions, there were $10^{0.2}$ and $10^{0.3}$ reductions of infectivity, respectively, with the cell-containing samples as compared to the control. These differences are not considered to be of sufficient magnitude to permet any conclusions, but do suggest the possibility that the infective viral particle does not have a direct function in the hemagglutination of IBV under the conditions of the test (Table XXIII).

Ultrafiltration experiments were performed to determine whether the hemagglutinin of IBV 40-22 could be separated from the infective particle. Virus infected allantoic fluid which had been frozen and thawed was clarified by centrifugation at 4,500 rpm for 20 minutes at 4 C. The supernatant fluid was passed through a millipore filter having a 450 millimicron porosity and the filtrate was then passed through a filter having a 50 millimicron porosity.

When each filtration process was completed, the filter pad was removed from the holder, broken into several pieces, and placed in a screw cap vial containing Earle's solution. The vial was shaken vigorously to resuspend any residual virus on the pad. The filtrates and residues from the pad were tested for infectivity and HA activity (Table XXIV). Earle's solution containing a filter treated in the same manner as above had been shown to be non-toxic to

TABLE XXIII

INFECTIVITY TITERS OF IBV 40-23 AFTER HEMAGGLUTINATION

Dilution of Virus		Titer
1:40	Cell free supernatant fluid	2.5
	Control	2.5
1:80	Cell free supernatant fluid	2.5
	Control	2.7
1:160	Cell free supernatant fluid	1.7
	Control	2.0
	Undiluted Virus before Trypsinization	6.8
	Undiluted untreated virus incubated with red blood cells	7.5

TABLE XXIV

THE EFFECT OF ULTRAFILTRATION UPON SEPARATION OF THE INFECTIVE PARTICLE AND THE HEMAGGLUTININ OF IBV 40-22

	Volume of sample (ml)	HA Titer	Infectivity Titer
Virus clarified by centrifugation at 4,500 rpm	15.0	2560	5.8
Filtrate from 450 millimicron filter	14.1	1280	4.3
Virus retained on filter of 450 millimicron	10.0	80	0.0
Filtrate from 50 millimicron filter	9.5	0	1.0
Virus retained on filter of 50 millimicron	10.0	320	3.4

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The virus used for this experiment had an infectivity titer of $10^{5.8}$ and an HA titer of 2560 prior to filtration. The filtrate from the 450 millimicron filter had an infectivity titer of $10^{4.3}$ and an HA titer of 1280. Apparently no virus was retained by the pad since it was innocuous after being suspended in Earle's solution, but the HA titer was 80. The filtrate from the 50 millimicron filter had an infectivity titer of $10^{1.0}$, but without demonstrable HA activity. The residue from the pad had an infectivity titer of $10^{3.4}$ and an HA titer of 320.

A second sample of IBV 40-22 was similarly treated two weeks later. Before the pad was removed from the holder, the filter apparatus was inverted and Earle's solution was introduced into the delivery tube. The apparatus was rotated gently to wash any virus which might have been passed through the filter, but remained on the underside of the pad and on the filter apparatus. The apparatus was then returned to normal position and the washing collected. Infectivity and HA determinations were made on the filtrates, washings, and residues (Table XXV).

The original infectivity and HA titer of the second sample was $10^{5.0}$, and 2560 respectively. The filtrate from the 450 millimicron filter was $10^{4.6}$, whereas the HA titer was 160. The infectivity titer of the washing was $10^{2.8}$ and the HA titer was 80. The residue of the pad had an

TABLE XXV

THE EFFECT OF ULTRAFILTRATION UPON SEPARATION OF THE INFECTIVE PARTICLE AND THE HEMAGGLUTININ OF IBV 40-22

	Amount of material in ml	Amount of final material in ml	HA Titer	Infectivity Titer
Virus clarified by centrifugation at 4,500 rpm	20	20.0	2560	5.0
Filtrate from 450 millimicron filter	15	14.2	160	4.6
Washing from 450 millimicron filter	2	2.0	80	2.8
Virus retained on 450 millimicron filter		14.2	0	2.4
Filtrate from 50 millimicron filter	11	8.0	0	0.3
Washing from 50 millimicron filter	4	4.0	0	0.0
Virus retained on 50 millimicron filter		8.0	2560	4.0

infectivity titer of $10^{2.4}$ with no observable HA activity. The filtrate of the 50 millimicron filter was $10^{0.3}$ with no detectable HA activity. Infectivity and HA activity was not demonstrated in the washing. The residue had an infectivity titer of $10^{4.0}$ and an HA titer of 2560.

While the data from both experiments cannot be analysed objectively and correlated on a definite numerical basis, certain differences between viral infectivity and HA activity can be demonstrated with respect to an apparent role of each.

It is apparent that the majority of the virus, as represented by infectivity tests, passes rather readily through a 450 millimicron filter, but is retained by a 50 millimicron filter. In both experiments, the filtrates from the 50 millimicron filter had no demonstrable HA activity, and a low degree of infectivity. Both HA and infectivity were readily demonstrated with the filter which was resuspended in Earle's solution. While this data cannot be used for definite conclusion, they do suggest that HA activity of IBV induced by trypsinization of the virus may be independent of the infective viral particle.

The immunological response of chickens was studied with normal, trypsin-treated and heat-inactivated IBV.

Sera from four chickens receiving two inocula of a 0.1 ml via the trachea four weeks apart with IBV 41-9, which had been trypsinized at 37 C for three hours, had an

$ID_{50}NI$ of at least $10^{7.8}$ at the sixth week using untreated IBV 41-9 for the neutralization test. Sera from chickens receiving the same inoculum intravenously and weekly for the next five weeks showed an $ID_{50}NI$ of $10^{7.3}$ at the sixth week.

Virus inactivated at 56 C for two hours without trypsinization, and which was non-infectious for chicken embryos, was administered intravenously according to the schedule for chickens receiving trysinized virus by this route. The $ID_{50}NI$ was at least $10^{7.8}$ at the termination of the experiment.

Untreated virus was administered according to the schedule for intratracheal inoculations of trypsinized virus, and the $ID_{50}NI$ was $10^{7.0}$ at the sixth week.

The average $ID_{50}NI$ for control chickens was $10^{0.7}$ at the beginning and end of the experiment (Table XXVI).

The N.I. of the serum produced against intravenously administered trypsinized virus is $10^{0.5}$ lower than the N.I. of serum produced against intratracheally administered trypsinized IBV. The N.I. of the serum produced against heat inactivated virus is $10^{0.8}$ higher than the N.I. of the serum produced against an untreated virus inoculated intratracheally. Although there is a difference between the latter serum N.I. and the N.I. of the former sera, the significance of this value is uncertain. IBV 41-9, trypsinized at 37 C for three hours or heated at 56 C for 30 minutes, retains its antigenicity (Table XXVII).

TABLE XXVI

THE EFFECT OF MODIFIED IBV ON THE PRODUCTION OF NEUTRALIZING ANTIBODIES

Neutralization Indices of Pre-infection Sera

Serum	Serum Titer LD_{50}	Virus Titer LD_{50} of IBV 41-10	N.I.
		6.3	
Serum A	6.5		-0.2
Serum B	4.8		1.5
Serum C	5.2		1.1
Serum D	6.1		0.2

TABLE XXVII

THE EFFECT OF MODIFIED IBV ON THE PRODUCTION OF NEUTRALIZING ANTIBODIES

Neutralization Indices of Post Infection Sera

Serum	Serum Titer ID_{50}	Virus Titer ID_{50}	N.I.
Trypsinized Virus Intravenously	0.5	7.84	7.34
Trypsinized Virus Intratracheally	0.0		7.84
Heat Inactivated Intravenously	0.0		7.84
Untreated Virus Intratracheally	0.83		7.01
Control	7.43		0.41

IBV 41 and 42 were propagated in chicken embryo kidney cell culture with Hank's balanced salt solution, 0.5 percent lactoalbumin hydrolysate, and two percent bovine serum. At 48 hours when cytopathogenic effect was distinct, the extracellular fluid was harvested. The untreated virus infected cell culture medium showed no HA. When infected cell culture fluid was processed exactly as infected allantoic fluid, the HA titer for both strains of virus was 160. The fluid from uninfected cell cultures treated in the same fashion was 40.

Several tests were performed to determine if hemadsorption of IBV would occur in a manner similar to influenza when the red blood cells are added to infected cell cultures. To chicken embryo cell cultures inoculated 24 or 48 hours previously with IBV 41, 0.2 ml of a 0.4 percent suspension of red blood cells was added to the nutrient medium and incubated for one minute. The same procedure was followed after removal of the nutrient medium. Hemadsorption was not observed.

In other tests, the nutrient medium was removed and either 0.25 or one percent trypsin solution was added for various intervals ranging from 30 seconds to five minutes. The cells were washed with saline, and 0.2 ml of a 0.4 percent suspension of red blood cells were added. Hemadsorption was not observed. After five minutes in contact with trypsin, the cells sloughed from the wall of the tube.

Periodate was tested to determine its effect on the HA of IBV. To two volume of infected allantoic fluid was added one volume of sodium periodate, 0.1 to 0.001 M. After incubation at 37 C for one, two, and three hours, the reaction of periodate was stopped by the addition of one volume of a one percent glycerol-saline mixture. HA could not be detected by this method.

Heat alone will not induce HA.

Freon and n-heptane, one volume, was added to two volumes of virus infected allantoic fluid before or after trypsinization of IBV 40-23 at 37 C for three hours.

TABLE XXVIII

THE EFFECT OF FREON ON HEMAGGLUTINATION OF IBV 40-23

Treatment	HA Titer
Trypsinized Virus	5120
Freon treatment before trypsinization	5120
Freon treatment after trypsinization	0
Freon treatment alone	0

Freon alone did not induce HA and was without effect when used before trypsinization, but negated HA activity when used after trypsinization of the virus.

The receptor destroying enzyme of V. cholerae

destroys the cellular receptor sites to which the myxoviruses adsorb. The standard virus for titration of RDE is the PR8 strain of influenza. The RDE is serially diluted in calcium borate buffer. After incubation with a one percent suspension of cells for 30 minutes at 37 C, ten units of virus is added and the mixture reincubated for 30 minutes. The end point is the lowest dilution of RDE in which HA is inhibited.

TABLE XXIX

TITRATION OF RDE USING PR8 AND IBV 17-40

Time (Minutes)	Inhibition of HA Titer of PR8	17-40
30	40	20
45	80	80
60	80	80

The magnitude of inhibition of HA by RDE was essentially the same with either PR8 or IBV 17-40 at the several test intervals. The only difference was at 30 minutes where the titer was 40 for PR8 and 20 for IBV. From these data, it would seem that the cell receptor site utilized by IBV are similar to those used by influenza.

Definite separation of naturally occuring non-specific and specific inhibitors of the hemagglutination test has not been accomplished for the utilization of trypsin modified

IBV in the assay of antibody.

The following treatments of normal and anti-IBV serum did not influence the inhibitory properties of the sera:

1. 56 C for 30 minutes
2. 62 C for 20 minutes
3. filtration through a Seitz EK filter
4. one percent trypsin and five drops of toluene for three hours at 37 C followed by 100 C for two minutes
5. toluene alone plus 62 C for 20 minutes
6. trypsin in concentrations of 0.8 or 16 mg/ml, at 37 C or 56 C for 30 minutes to three hours
7. sodium or potassium periodate, 0.9 to 0.01 M at 4 C overnight, 37 C for three hours, or 56 C for 30 minutes
8. zymosan in concentrations of one, two, five, ten or 20 mg/ml had no effect on the inhibitor, indicating that properdin was not the inhibitory agent
9. equal volumes of RDE and sera

A phosphate buffer, pH 8.2, containing 8 mg of trypsin 1 ml and M/90 potassium periodate removed the inhibitory properties of both negative and anti-IBV sera. Without such treatment the inhibitory property of both sera is the same. This treatment offers perhaps the most fruitful approach for detection of specific antibody as it is the only one in which removal of the inhibitor has been demonstrated.

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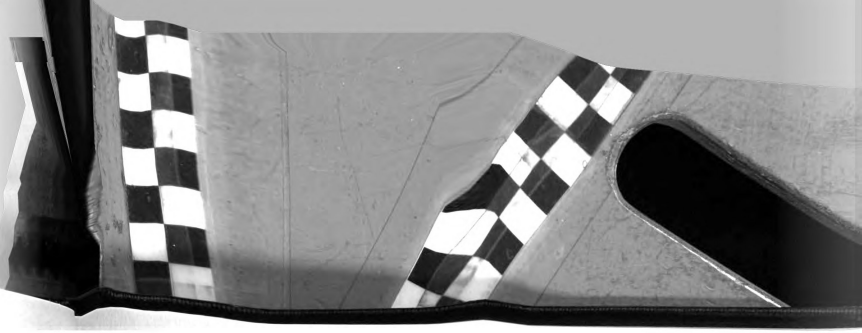
DISCUSSION

The mechanism of the induction of hemagglutination by IBV must be determined if an hemagglutination-inhibition test is to be developed using trypsin modified virus-infected allantoic fluid. Trypsin is a relatively specific enzyme which can act as a peptidase or an esterase, when an L-arginine or L-lysine residue contributes to the carboxyl group[120]. Attention must then be directed to the location of the substrate upon which trypsin reacts.

The hemagglutinin of the myxoviruses[58,59] and the arthropod-borne[96] viruses is the virus particle itself. When influenza strain PR8 is inoculated into embryonating chicken eggs via the allsntoic cavity, the maximum infectivity titer is reached at the ninth hour post inoculation, remains fairly constant until the 20th hour, followed by a gradual loss of activity. The HA titer gradually rose to a maximum titer at the 20th hour and remained constant until the termination of the experiment 40 hours after inoculation[106].

Mumps virus inoculated via the allantoic cavity could first be detected 48 hours after inoculation. Hemagglutinating activity was first detected after 96 hours. Maximum infectivity was observed at the 120th hour post inoculation, whereas HA activity is maximum at the 144th hour. The 24

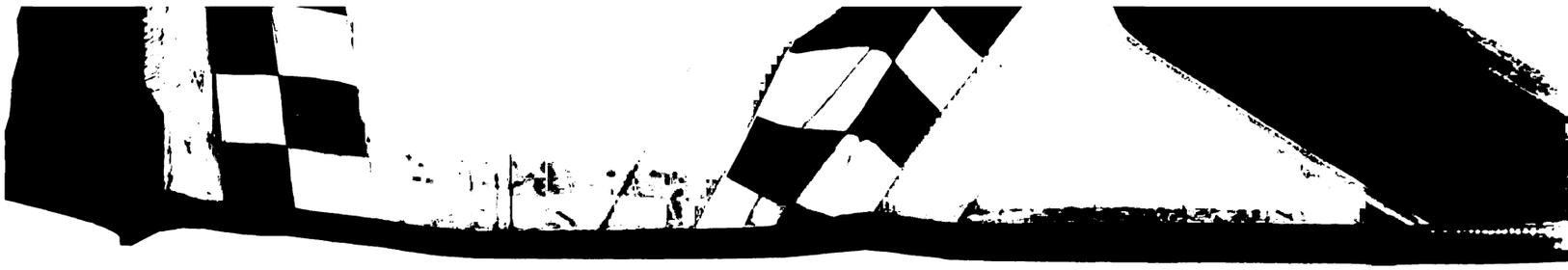


hour difference between the observance of maximum infectivity and maximum HA activity is considered to be of no significance. It was concluded that log $10^{4.3}$ EI_{50} or higher was necessary for HA to be detected[45]. A figure of about log 10^{6} ID_{50} is given for one HA unit of influenza virus[38].

Growth curves of the egg-adapted strain of IBV in eggs inoculated via the allantoic cavity shows a maximum titer at 12 hours post inoculation. Early egg passage virus enters the log phase in six hours and a maximum concentration is attained within 24 to 30 hours[64]. The Showa-machi strain of IBV in the 40th egg passage reaches a maximum infectivity titer at approximately 16 hours after inoculation[100].

Infectious bronchitis virus 17-40, 40-23, and 41-6 inoculated into embryonating chicken eggs via the allantoic cavity, showed maximum infectivity 24 hours post inoculation. The infectivity titer decreased during the next 24 to 48 hours, after which the activity remained fairly constant to the 101st hour. The hemagglutinin of the trypsin modified IBV 41-6 decreases in activity 24 hours post inoculation. At this time, the HA activity of 17-40 and 40-23 was not detectable. At the time when the infective particle entered the stationary phase, the HA activity was detected.

If the HA activity of IBV is an intrinsic property of the infectious particle, there should be an increase in HA titer corresponding to a rise in the infectivity titer. Since such a relationship is not observed, it is concluded



that another mechanism is operative.

Further evidence indicates that the hemagglutinin is not the infective particle. Chickens were inoculated intravenously with trypsin modified virus, and intratracheally with untreated and treated virus. When neutralization indices were calculated, the differences were not great enough to conclude that trypsin so modified the infective viral particle that a specific antibody was produced.

There are two phenomena reported in which the hemagglutinin was associated with the virus particle with no relation between the HA titer and the infectivity titer. Under certain conditions of inoculation of influenza virus, the allantoic fluid will yield high titers of noninfectious hemagglutinins as well as normal infectious particles which will participate in HA[118]. The noninfectious particles lack a complete complement of ribonucleic acid[1]. It has been suggested that the infected cell produced more protein than nucleic acid and thus noninfectious as well as infectious particles are produced[67].

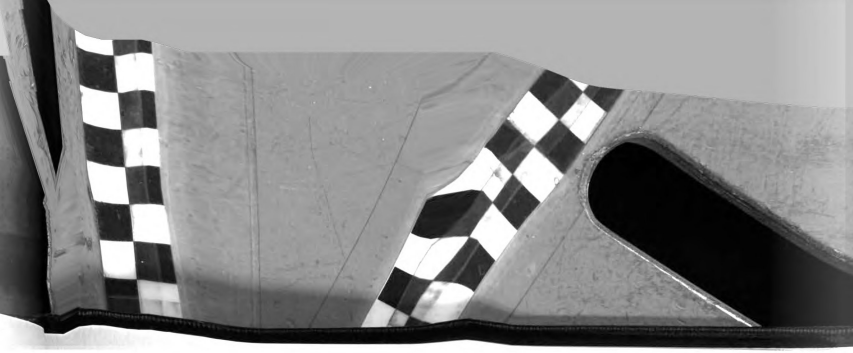
The other possibility would be the presence of an inhibitor of HA activity which would mask any correlation between infectivity and hemagglutinating activity. An inhibitor of HA of influenza virus was found to exist in the allantoic fluid[111]. The virus in the active state is capable of destroying the mucoid inhibitor and HA will result. Indicator viruses are not capable of destroying the

mucoid, but HA will occur when the inhibitor is destroyed by trypsin, periodate, or RDE.

The lack of correlation between the HA activity of IBV and the infectivity cannot be explained by von Magnus' description of the incomplete form of influenza. Rather, the reduction or disappearance of hemagglutinating activity would indicate that more nucleic acid is produced than is protein, or a necessary antigen is not being incorporated into the viral particle. If this were so, then the HA activity would be intimately associated with the infective particle at the time of appearance.

Data were collected which indicated that the infectivity titer of IBV after hemagglutination is of the same magnitude as that which had not been reacted with cells. This would indicate that the infective particle does not significantly contribute to HA.

If a mucoid inhibitor were masking any correlation between infectivity and HA, it may be destroyed by either active virus or trypsin . It is unknown whether IBV can destroy the mucoid inhibitor. However, since this virus is stored in a frozen state or handled for short periods of time at temperatures not exceeding 5 C, the enzymatic activity of the viral particle is not expected to be very great. The conditions employed for the trypsinization of this virus to induce HA favor the formation of an indicator virus, which of its nature cannot destroy the inhibitor.

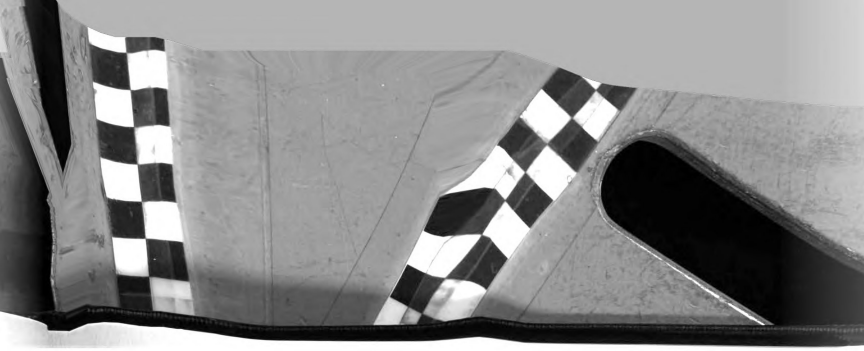


Nevertheless, trypsin should completely or partially inactivate the inhibitor which would result in detection of HA. Since trypsin induces HA in later harvests of infected allantoic fluid, it seems that the answer is not the presence of an inhibitor unless conditions change that result in the destruction of the inhibitor. One condition may be longer exposure to IBV. Thus, this possibility may not be neglected.

Another mechanism which may be operative is that the hemagglutinin of IBV is distinct from the infective particle, similar to the viruses of the pox and psittacosis group. If this were the case, the growth curve and HA curve for IBV need not show a direct relationship. In support of this hypothesis is the fact that viral infectivity, but not HA activity, can be detected after passage through a 50 millifilter. Also lending support to this hypothesis is the fact that trypsinized infective particles are not absorbed to red blood cells.

It is concluded from the results of infectivity and HA determinations, ultrafiltration, and infectivity after HA that the HA activity of IBV infected allantoic fluid is a property of an antigen distinct from the virus particle. Of the various agents tested, trypsin is the only one which will consistently cause induction of HA by IBV suspended in allantoic fluid[25].

Trypsin reacts with a normal constituent of allantoic



fluid to cause HA. When ETI is added, the non-specific HA is eliminated. The ETI does not effect the HA of virus infected allantoic fluid. It seems possible that trypsin is reacting with a "specific" and "non-specific" agent to induce HA. Hemagglutination is evident only with the "specific" agent after the addition of ETI. The equations for such a reaction may be:

1. (Substrate) + (Trypsin) $\rightleftarrows$ HA + (ETI) $\rightarrow$ no reaction
2. (Substrate) + (Trypsin) $\longrightarrow$ HA + (ETI) $\rightarrow$ HA
3. (Trypsin) + (ETI) $\rightleftarrows$ (Trypsin-ETI)

Therefore, ETI is necessary for the specificity of the test, but the exact role is unknown. It may simply be that all equations are reversible except number 2. It can be seen from the above reactions invloving ETI that either the non-specific substrate is absent in virus infected allantoic fluid or, if present, does not play a significant role when the specific substrate is present.

In the consideration of this mechanism, the ETI probably is competitive in nature, and all reactions are dependent on the concentration of inhibitor, enzyme, and substrate. This fact is clearly seen from the data obtained using various amounts of trypsin and ETI. Maximal activity is detected when both reagents are at a one percent concentration. Activity is not detected at 0.01 percent concentration. That higher titers are observed when the concentration of trypsin is ten times greater than ETI may be a reflection

of the non-specific hemagglutinating agent. When the concentration of ETI is ten timesgreater than trypsin, the lack of detectable HA activity may be a reflection of the dependence of the reaction on concentration.

A means to elucidate the action of trypsin may be the employment of various antisera. Whether trypsin acts as a proteolytic enzyme or adsorbs onto an antigen distinct from the viral particle to induce HA, the specificity of the homologous antibody may be so altered as to show a difference in titer when compared to antibody produced against normal virus.

If chickens were inoculated intravenously with trypsin modified virus, antibodies homologous to the antigen should be produced. Trypsin modified virus and its specific antiserum in a HI test may yield different results as that when treated virus is employed with antiserum produced against untreated virus. A difference was observed in the serum neutralization test but was not regarded as significant.

The non-specific inhibitor of the HA of IBV present in normal and immune serum would have to first be removed. Since ether extraction and butanol separation failed to remove the inhibitor, it is concluded that the inhibitor is probably not lipoidal in nature. Heat and/or trypsin had no effect on the inhibitor. Trypsin and potassium periodate treatment of negative and anti-IBV sera results in the negation of any effect of inhibitors or specific antibody.

Perhaps by varying the concentrations of the reagent, the inhibitors may be removed without the destruction of the specific antibody. The possibility that the inhibitor is an enzyme which negates the action of trypsin or destroys the erythrocyte receptor site cannot be dismissed.

Further investigation may possibly determine the mode of action of trypsin either in two ways. If trypsin acted as a proteolytic enzyme, a new substrate should be produced by destruction of a surface moiety. The trypsin treated infected allantoic fluid could be subjected to various enzymes and if destruction of HA occurred with a certain enzyme reaction, the substrate would be partly defined.

The receptor site to which the myxoviruses attach during hemagglutination is destroyed by the action of RDE. This enzyme also destroys the mucoid inhibitor of HA of this group of viruses.

The receptor destroying enzyme destroys the receptor site on the erythrocyte to which the hemagglutinin of IBV adsorbs in the process of HA, indicating that the same receptor site is possibly used by the myxoviruses and IBV. RDE activity is destroyed by trypsin and the IBV sample employed in this test may have active trypsin present. The activity of the RDE on the red blood cell is completed before the trypsin is added and the conclusion is valid. Nevertheless, equal volumes of RDE and serum will not remove the inhibitor of HI.

A property which has been frequently employed in characterization of a virus is the thermostability of the infective particle and the hemagglutinin.

The hemagglutinin of the influenza strain PR8, which is the prototype of the myxoviruses, does not follow a first order rate of inactivation at 61 C. A straight line is obtained when $1/\sqrt{\log C}$ is plotted against time which is a three halves order reaction. After ten minutes at this temperature, the HA activity was almost completely destroyed[75].

The thermostability of the hemagglutinin of the LEE strain of influenza type B, and the Weiss and PR8 strain of influenza type A was studied at 61.5 C. The Weiss strain was almost completely inactivated in one and one half hours, and the PR8 in six hours. However, the LEE strain showed no significant loss of titer in eight hours at this temperature, indicating the great variation in stability among these strains[99].

The hemagglutinins of influenza, PR8, and LEE are stable at 55 C for one hour, but a significant reduction of titer occurred within 15 minutes at 60 C[59].

Variations in the thermostability of hemagglutinins have been observed among various strains of Newcastle disease virus[54].

Infectious bronchitis virus subjected to 56 C gave results similar to those obtained with the LEE strain of

influenza B. There were no differences among the various strains of IBV. All strains tested were stable for at least three hours , and some were tested at nine hours with no reduction of HA titer. However, there is some evidence presented which indicates a variation of the hemagglutinating activity of various strains of IBV when subjected to trypsin at 56 C. Further study must be done to validate these preliminary findings.

The hemagglutinin of influenza virus is more heat stable than the infective viral particle[59]. The rate of inactivation of a virus may proceed according to the kinetics of a first order reaction, as in the case of the PR8 strain of influenza[76]. Inactivation rates of other viruses, such as murine polio[7] and human polio[123], show deviations from the normal curve, suggest a more complex reaction than described by first order kinetics[7]. Infectious bronchitis virus follows a bimodal rate of inactivation which can be studied as a three halves order reaction[104]. The data collected in the present thermostability study confirms this conclusion.

Viral infectivity, but not antigenicity, was destroyed within 90 minutes at 56 C. The neutralization index of serum from chickens inoculated intravenously with virus heated at 56 C for 120 minutes was comparable to the index of serum from chickens inoculated intratracheally with untreated virus. The HA activity was not destroyed after

nine hours at this temperature. These differences emphasize the variety of chemical composition of the various antigens. Probably, the infectivity of IBV is associated with the nucleic acid portion of the viral particle. This property of nucleic acid was first demonstrated with tobacco mosaic virus[41]. The antigenicity of IBV may be associated with the viral particle and perhaps proteineous. The antigenicity of the fowl plaque virus resides on the protein coat of the virus particle[101]. The thermostability of this neutralizing antigen may be of some consequence in the preparation and storage of vaccines.

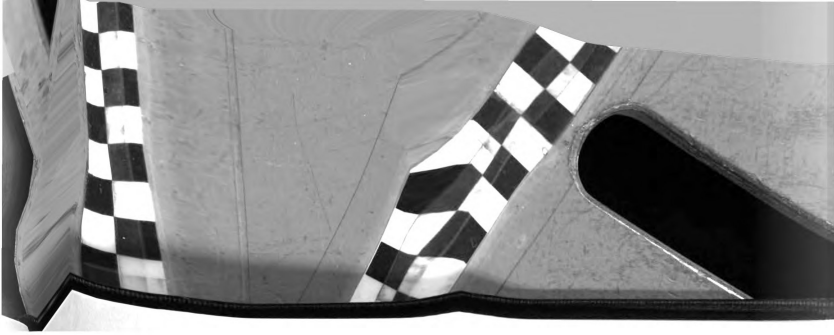
The effect of trypsin on viral infectivity has been studied with a number of different viruses. With certain viruses as feline pneumonitis and the GD VII strain of murine encephalitis, the infectivity titer will increase due to the dissociation of aggregates[73]. This enzyme has been used in the preparation of virus for electromicroscopy by destruction of amorphous material[94]. The LEE strain of influenza, and potato virus X are inactivated by trypsin. After three hours at 37 C, the infectivity titers of trypsinized IBV 41-7 and 3-16 were significantly lower than that of the controls. The titer of the trypsinized sample of IBV 40-23 was approximately the same as the control. The mechanism of inactivation is presumably one of digestion of protective proteineous material. It may either be the destruction of an antigen necessary for replication of the particle, or the unfolding

of a peptide which would render the nucleic acid more susceptible to heat.

After 12 hours of incubation, the trypsinized sample of IBV 3-16 has a greater titer than the untreated control. The same has occurred at 20 hours with IBV 41-9. This may be an artifact or it may be that the enzyme has been denatured by heat and is now acting as a protective colloid. After 20 hours at 37 C with trypsin, one sample of IBV 3-16, and IBV 41-9 still retained some infectivity in both the trypsinized and untreated sample.

Disruption of viral aggregates may have occurred with some samples as the titer of trypsinized IBV 3-16 was ID_{50} $10^{0.7}$ greater than the control. In some cases no effect was observed. The question concerning the disruption of viral aggregates in allantoic fluid could be studied by means of a sonic oscillator.

In studying enzymatic digestion, the concentration of extraneous material must be considered. The lag period observed in the infectivity titrations of IBV 41-7 and 3-16 may be due to the destruction of soluble material which were in higher concentrations than the infective particle, and only after the unaltered enzyme was released from this substrate did it react with the virus particle. This lag may be due to the digestion of an inhibitor coating on the infective particle much like that which has been proposed for influenza[20]. It has been shown that trypsinized virus

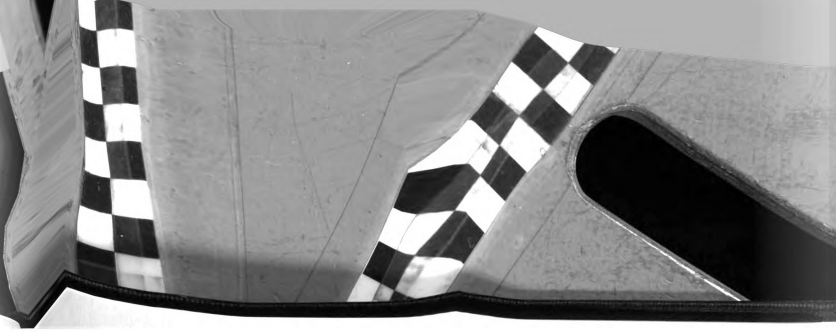


administered intravenously will stimulate the production of antibodies to the same level as untreated virus given intratracheally.

Any system involving the agglutination of red blood cells is hampered by the variation found in the reagents used. One of the variations which must be considered is the reactivity of the erythrocyte. HA titers of vaccinia varied considerably depending on the species of fowl cells employed. Cells from only 50 percent of donors tested were reactive in the HA tests and this proportion is dependent on the age of the donor animal[14,24]. Another factor which will influence the HA titer is the length of storage time of drawn cells at 4 C.

Such variables are found when the HA activity of IBV is determined. Erythrocytes from different chickens will not react with a given sample to give the same titer. Erythrocytes collected in Alsever's solution could be used to determine HA activity of trypsin treated allantoic fluid, but they were unsuitable for use after one week of incubation at 4 C.

In a preliminary experiment chickens were hatched, and reared in isolation. Some birds were infected with IBV. Reactivity of erythrocytes is apparent in birds which were three weeks or older. The activity of the cells from infected birds was the same as those from birds free of IBV. In a second experiment using different birds, it was again

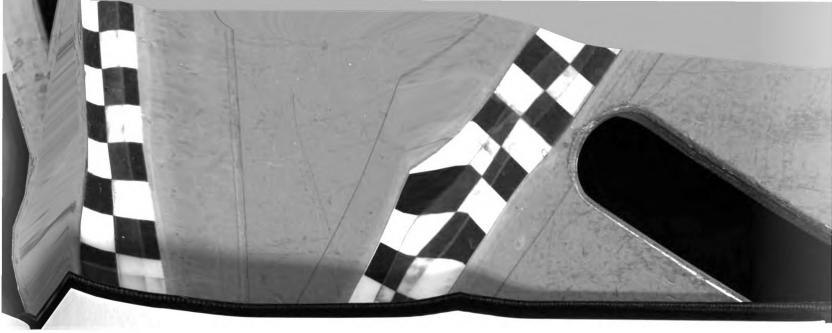


found that HA activity could not be demonstrated until the birds were three weeks old. The data of this experiment paralleled the previous results which indicate that previous exposure to the virus does not increase the reactivity of the erythrocytes. Nevertheless, the data shows an alteration of the receptor site three weeks after hatching, which indicates the site is absent, blocked, or non-reactive. Since exposure to virus has no effect on the development of the receptor site, all variations in reactivity were merely reflections of the intrinsic variation of the erythrocytes.

SUMMARY

1. Hemagglutination titers of various cultures of infectious bronchitis virus after treatment with one percent trypsin at 56 C for 30 minutes are comparable to those at 37 C for three hours. Eggwhite trypsin inhibitor is added after incubation. The former procedure has been selected for the standard incubation period for the trypsin modified hemagglutination test of infectious bronchitis virus.
2. The hemagglutinin of infectious bronchitis virus can be demonstrated only with erythrocytes from chickens and turkeys.
3. Erythrocytes from chickens less than three weeks old are not reactive to the hemagglutinin of infectious bronchitis virus.
4. Reactivity of erythrocytes varies among donors. Frequent bleeding may result in the loss of reactivity of the erythrocytes.
5. Trypsin modified infectious bronchitis virus is stabile for hemagglutination for three weeks at -65 C, but fluctuations in titer occur during the next four weeks.
6. After six and one-half hours at 56 C, prior to trypsinization, infectious bronchitis virus retains its ability to agglutinate erythrocytes.

7. After two hours at 56 C, the virus is non-infectious for chicken embryos; but it is antigenic for chickens as shown by antibody response measured by serum neutralization tests.
8. There is a reduction of viral infectivity when incubated with trypsin at 37 C.
9. With infectious bronchitis virus 17-40, 40-23, and 41-6 the maximum viral infectivity for embryos and the hemagglutination titer of infected allantoic fluid is attained at 24 and 72 hours, respectively, after inoculation.
10. Infected allantoic fluid after hemagglutination shows no difference in infectivity as compared to similarily treated controls which were not reacted with chicken erythrocytes.
11. Embryo infectivity and hemagglutinating activity of infectious bronchitis virus 17-40 and 40-22 can be demonstrated in filtrates from 450 millimicron Millipore filters. Filtrates from a 50 millimicron filter are infectious for chicken embryos but do not possess hemagglutinating activity.
12. The same endpoint is attained with infectious bronchitis virus 17-40 and influenza virus strain PR8 when 10 hemagglutinating units of each are reacted with chicken erythrocytes previously treated with receptor destroying enzyme of Vibrio cholerae.



13. Hemadsorption does not occur with infectious bronchitis virus 41 cultivated in and producing cytopathogenic effects in chicken embryo kidney cell cultures.
14. Extracellular fluids from infectious bronchitis virus 41 and 42 cultivated in chicken embryo kidney cell cultures show a hemagglutination titer of 160 as compared to 80 for uninoculated cells.
15. Various methods for the removal or destruction of the non-specific inhibitor of hemagglutination present in normal serum proved ineffective.

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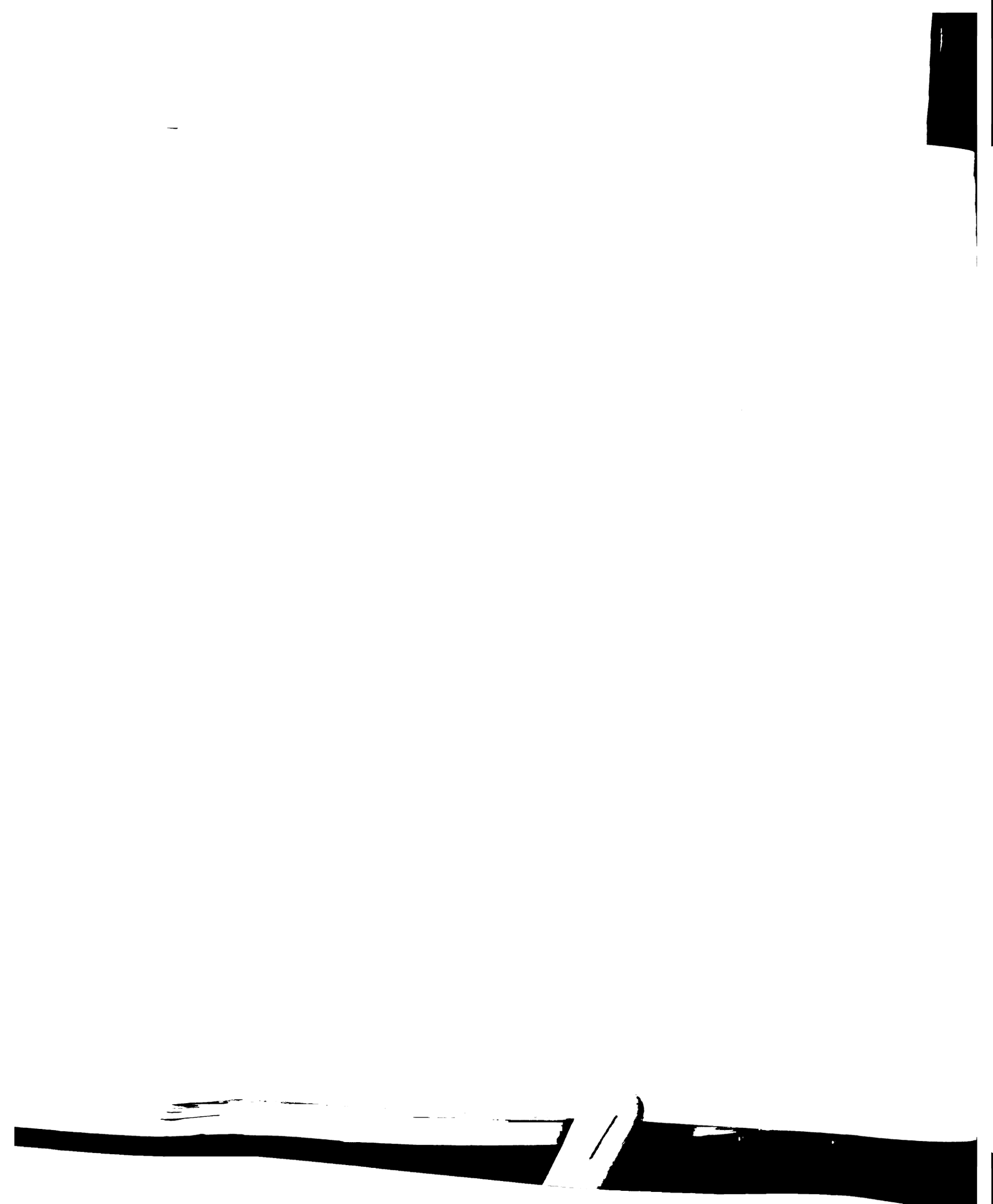

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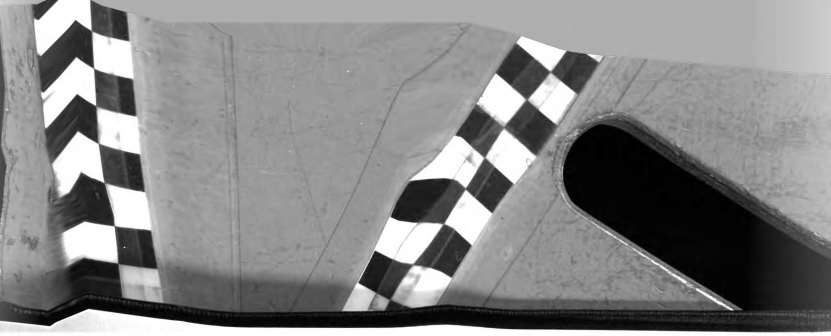

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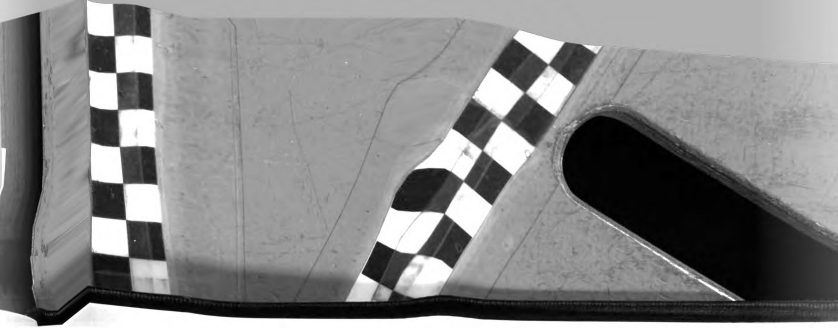

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