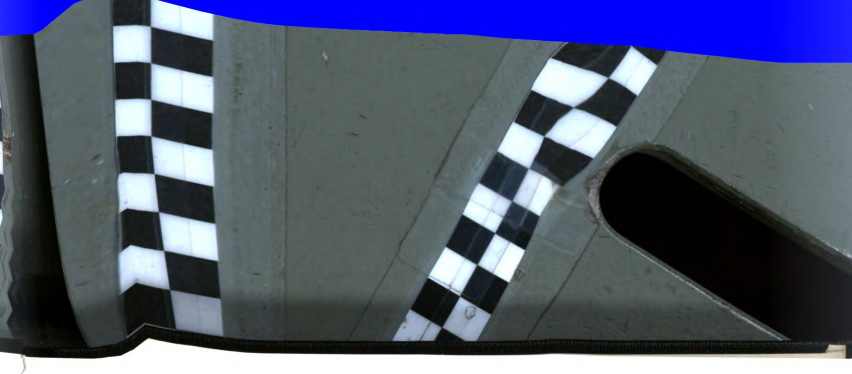

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HEMAGGLUTINATION BY TRYPSIN-MODIFIED INFECTIOUS BRONCHITIS VIRUS

By

LEO JOHN CORBO

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

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

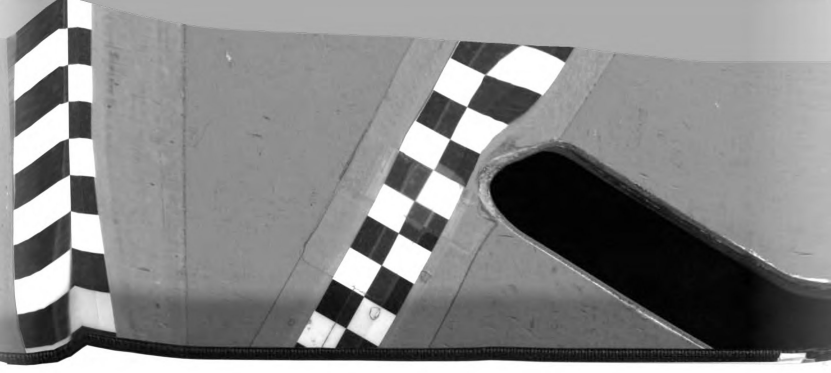
1957

This work is

respectfully dedicated

to

MY FAMILY



ABSTRACT

Infectious bronchitis of chickens (IB) is an acute and highly contagious disease of economic significance to the poultry industry.

The purpose of the present study was to determine if the serological techniques employed with the causative virus, Tarpeia pulli, could be expanded to encompass hemagglutination. Infectious bronchitis virus (IBV) does not agglutinate red cells, and the only serological test which is applicable is the serum-neutralization test.

Twelve strains of IBV, one of which was completely egg-adapted, were tested. Newcastle disease virus (NDV) and normal allantoic fluid (NAF) were used for controls.

All virus was in the form of infected allantoic fluid of embryonating chicken eggs collected on the twelfth day of incubation. Newcastle disease virus and the egg-adapted strain of IBV were inoculated on the tenth day, whereas the other strains of IBV were inoculated on the ninth day. Normal allantoic fluid was collected on the twelfth day, also.

Induction of a hemagglutinative agent in IBV was attempted through utilization of the proteolytic enzymes, trypsin, pepsin, papain, and rennin. Trypsin proved effective.

A standard method of treatment (standard T process) was established. This consisted of mixing 1 ml of 1 per cent trypsin with 2 ml of the sample. The system was incubated for three hours at 37°C. and then 1 ml of eggwhite trypsin inhibitor was added. The complete system was then tested for hemagglutinative activity at 23°C.

All strains of IBV except the egg-adapted strain responded to treatment as evidenced by consistently high hemagglutination titers obtained when using chicken erythrocytes. Normal allantoic fluid and NDV were not affected by this treatment as NAF exhibited no hemagglutinative activity and that of NDV was unchanged.

A 98 per cent decrease in viral infectivity accompanied tryptic modification of IBV.

Adsorption of the hemagglutinative agent of modified IBV is rapid and complete at 37° and 23°C. but not complete at 4°C.

Modified IBV has not been successfully employed in hemagglutination-inhibition tests. Normal chicken serum and anti-IB serum inhibit hemagglutination.

The IBV hemagglutinin is distinguished from that of NDV and the nonspecific hemagglutinin of trypticase and tryptone on the basis of reactions with sera.

Spontaneous elution of the hemagglutinative agent of modified IBV is slight at 37° and 23°C. and more pronounced but not complete at 4°C.

Hemagglutination by modified IBV occurs when chicken red cells are employed. No agglutination of the red cells of the dog, cat, rabbit, cow, sheep, horse, or human type O has been demonstrated.

A direct correlation was observed between the optical density of the modified IBV system, the hemagglutination titer, and the infectivity titer.

It was demonstrated that the action of trypsin was directed to the virus, and not the chicken erythrocytes, for trypsin-modified red cells were not reactive.

ACKNOWLEDGMENTS

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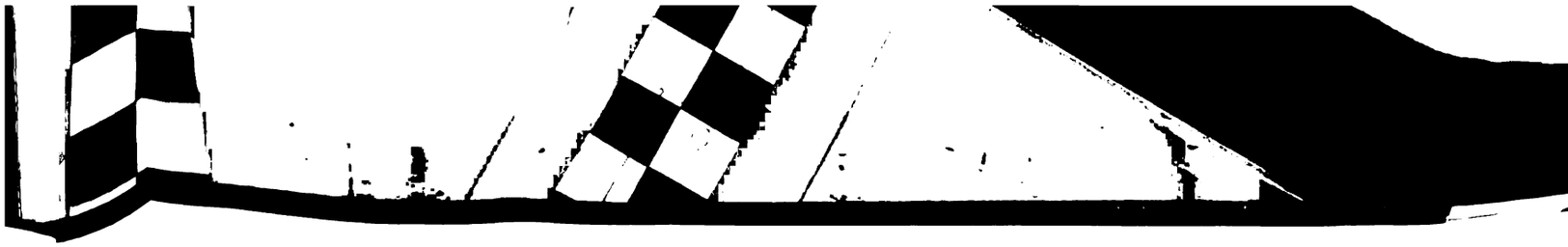


Leo John Corbo

candidate for the degree of

Doctor of Philosophy

Final Examination: May 15, 1957, 8:00 A.M., Room 101, Giltner Hall.

Dissertation: Hemagglutination by Trypsin-modified Infectious Bronchitis Virus.

Outline of Studies:

Major subjects: Virology and Bacteriology.

Minor subject: Chemistry.

Biographical Items:

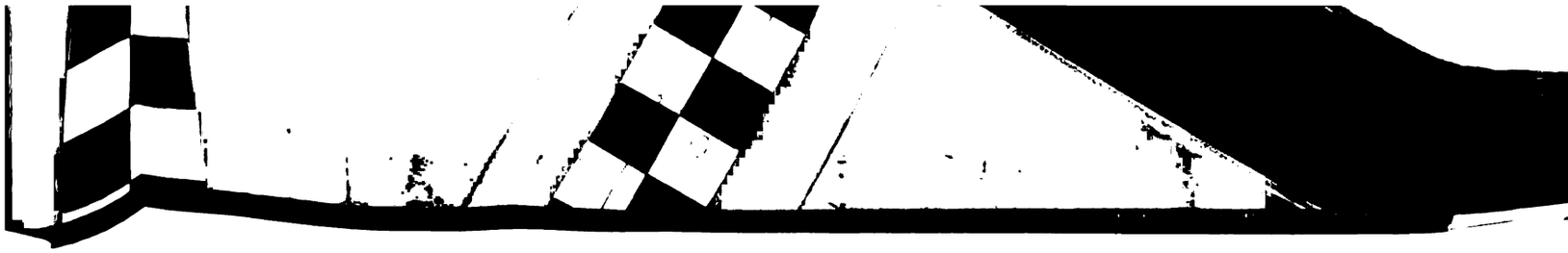
Born December 12, 1922, Elizabeth, New Jersey.

Undergraduate Studies: University of Notre Dame, 1940-1942, 1946-1947, 1950-1951.

Graduate Studies: University of North Carolina, 1951-1952; Michigan State University, 1955-1957.

Experience: Bacteriologist, Lobund Institute, Notre Dame, Indiana, 1952-1955.

Society Affiliations: Society of American Bacteriologists, American Public Health Association, American Association for the Advancement of Science, Society of Sigma Xi, The New York Academy of Sciences.



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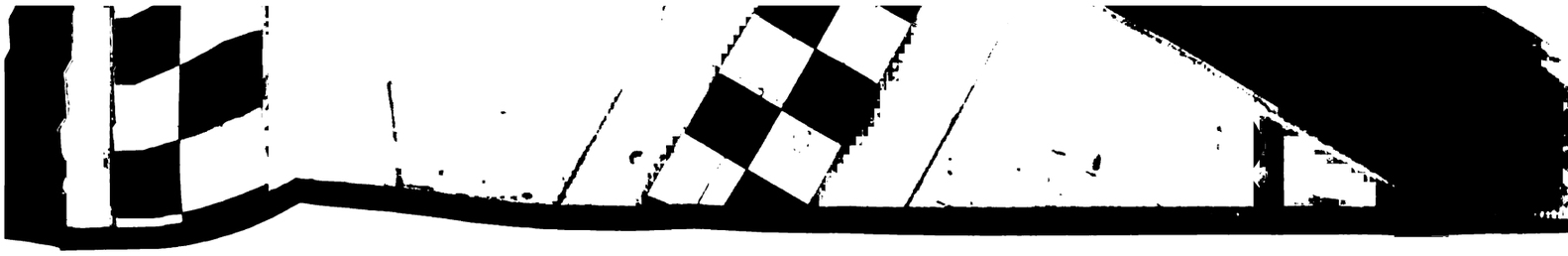

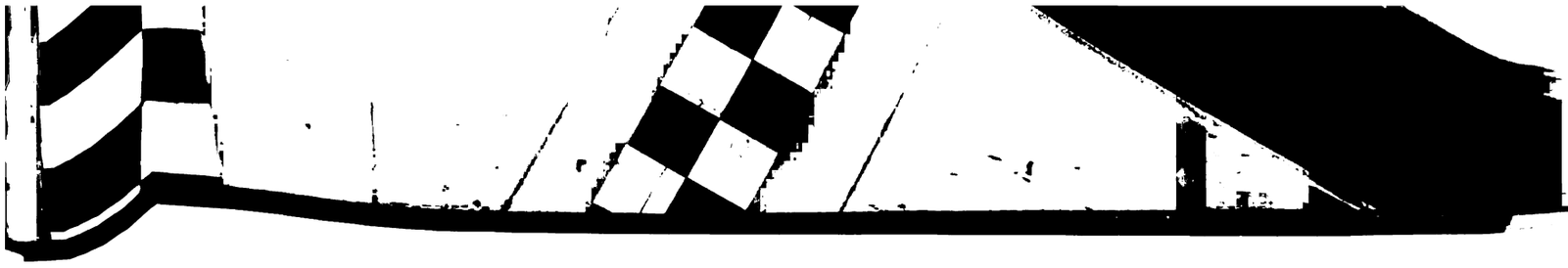

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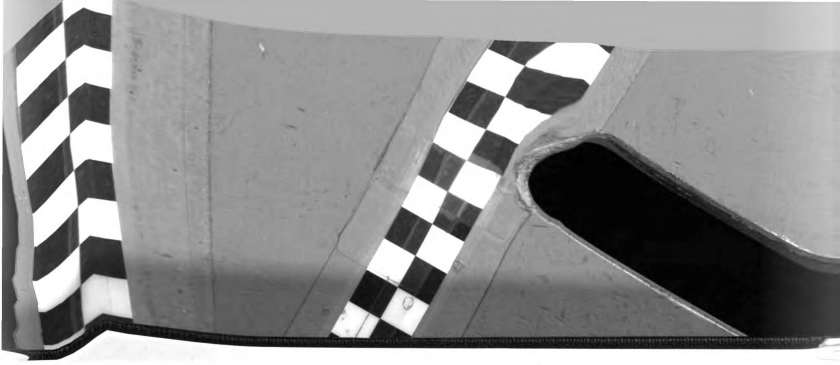

INTRODUCTION

Infectious bronchitis is a disease economically important to the poultry industry. The serum neutralization test is the only serological procedure that may be employed for diagnosis of the disease.

The object of the present study is to determine the potential of infectious bronchitis virus as a hemagglutinative agent.

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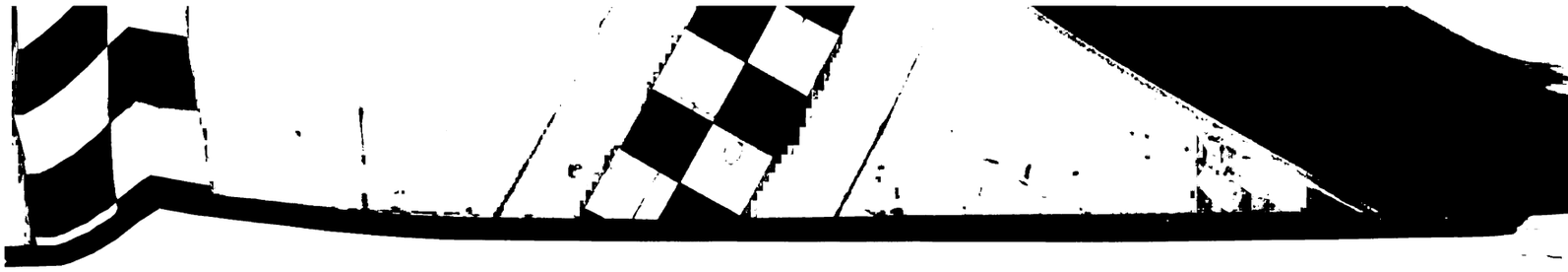


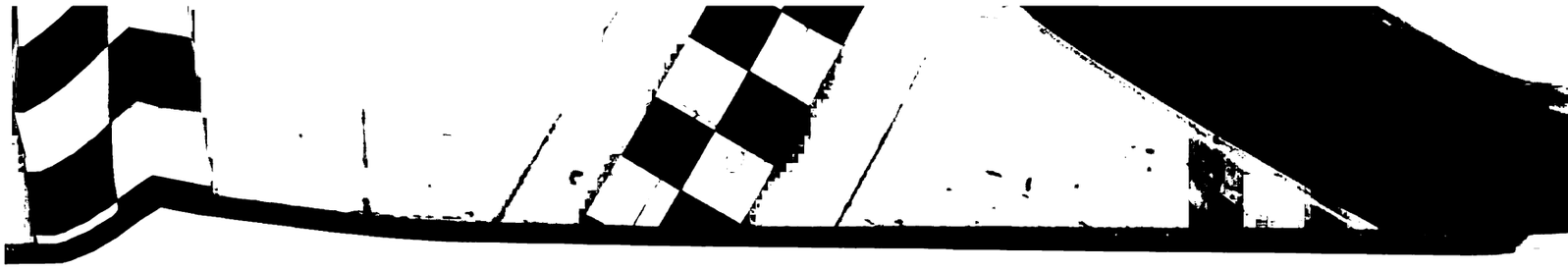
HISTORICAL REVIEW

Infectious Bronchitis

Infectious bronchitis (IB), an acute and highly contagious respiratory disease of chickens, was first described by Schalk and Hawn (87) in 1931 in North Dakota. Since that time the disease has been recognized throughout the United States, in Canada, Great Britain, the Netherlands, Japan, and Brazil (54, 94).

The causative agent is the virus Tarpeia pulli, which is spherical with filamentous projections and has a mean diameter of 70 mμ as determined by electron microscopy of the free elementary bodies (82, 83).

Infectious bronchitis virus (IBV) within the cells of the chorioallantoic membrane, has a mean diameter of 178 mμ and is devoid of filaments. Some virus particles exhibit a discrete internal structure which forms a pattern that suggests the possibility of a macromolecular arrangement within the elementary bodies. Rows of clear spots of less electron density, about 20 mμ in diameter, each of which contains a smaller, electron-dense body, make up this pattern (37).



The disease apparently is limited to chickens, and affects all ages, breeds, and sexes. Natural transmission is by direct contact or by aerosol. Experimentally, the disease may be transmitted readily following intratracheal, intranasal, and air sac inoculation (7, 35), but not by subcutaneous or intramuscular routes. Signs usually appear 24 hours after intratracheal inoculation. The most prominent signs are gasping, sneezing, coughing, and tracheal rales (5, 6, 34, 59).

Isolation of the virus. IBV can readily be isolated from lung and tracheal material collected during the incubation period and throughout the respiratory phase of the disease. Chicken embryos are inoculated with these specimens and the outstanding gross alterations produced by the virus are a curling and dwarfing of the embryo (7, 35, 38, 69). These lesions are pathognomonic of infection with IBV (69). Microscopic alterations include proliferation of mesodermal and ectodermal cells, edema of the amnionic membrane, necrosis and hemorrhage of the liver, interstitial nephritis, congestion of the spleen, and slight capillary congestion of the brain.

Beaudette and Hudson (7) and Delaplane and Stuart (36) observed that following chorioallantoic membrane inoculation there is but slight embryo mortality in early passage of the virus. With

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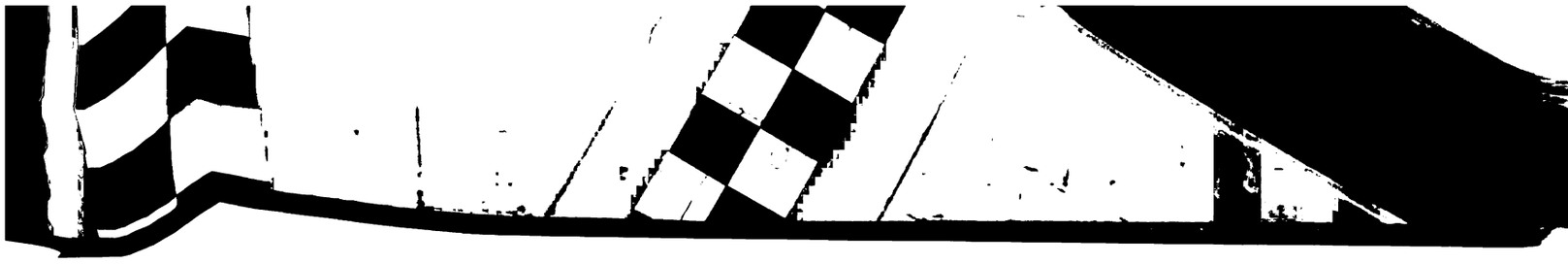

successive embryo passage the virulence of the virus increases as manifested by higher embryo mortality rates. Completely egg-adapted virus kills all embryos within 48 hours after inoculation (28). Complete adaptation varies with different strains. With some this has been observed at the sixty-fifth passage, and at the ninetieth and one hundred twentieth passages with others.

Egg-adaptation is accompanied by loss of pathogenicity and antigenicity for chickens.

Diagnosis of infectious bronchitis. Diagnosis of IB is based on the following: (1) clinical features; (2) isolation and identification of the virus; (3) neutralization tests; (4) cross-immunity tests (25).

The ability of anti-IB serum to neutralize IBV was first reported by Beach and Schalm (5). Mixtures of virus and serum were not infectious for susceptible chickens. This method was later applied with embryonating chicken eggs.

Cunningham (24) reported the serum-neutralization indexes of normal birds, not previously exposed to IBV, would not be expected to exceed $10^{1.517} \pm 10^{0.0376}$ or thirty-six neutralizing doses.

IBV fails to agglutinate chicken erythrocytes, and the hemagglutination-inhibition test cannot be used to identify either the virus

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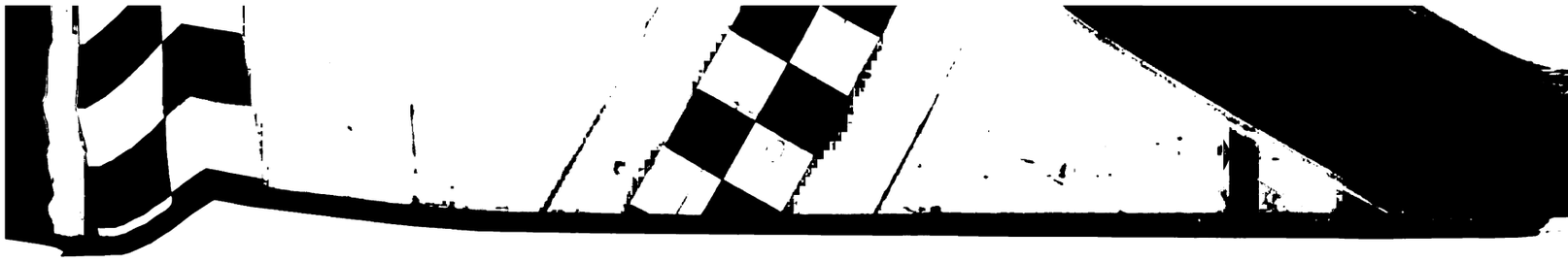


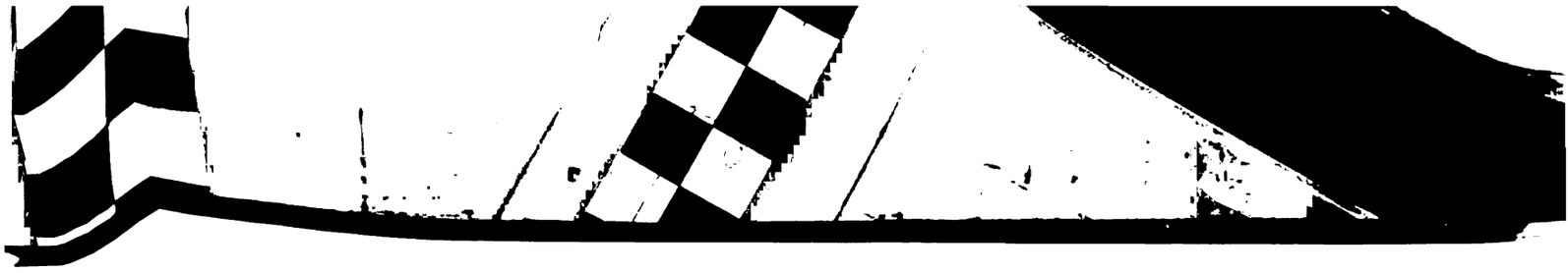
or specific antibodies other than by an elimination process (4, 25, 32, 33, 38).

Hemagglutination

Many substances such as plant (80) and animal (90) tissue extracts, the higher fungi (40), bacteria (80), rickettsia (81), pleuropneumonia-like organisms (40), and viruses (55) may agglutinate red blood cells of various species. Some of the reactions are only an observable phenomenon without an understanding of the mechanism of reaction, but others such as those with bacteria and viruses are specific.

Hemagglutination has been studied with many bacteria. Krauss and Ludwig (63) observed clumping of erythrocytes in the presence of staphylococci and vibrios. This was a direct bacterial hemagglutination, the mechanism of which remains to be elucidated.

Keogh, North, and Warburton (60) demonstrated adsorption of the polysaccharide antigen of Hemophilus influenzae on red cells and subsequent agglutination of the modified cells upon addition of homologous antibacterial serum. The same reaction has been observed with other bacterial antigens and antibodies. In general, polysaccharide rather than protein antigens are adsorbed by the untreated erythrocyte.



Assuming a two-step reaction in the modification of the red cell by polysaccharide antigen of various bacteria--(1) alteration of cell surface, and (2) attachment of antigen--Boyden (10) investigated a number of compounds to determine if they possessed the capacity to produce the first reaction and render red blood cells capable of adsorbing protein antigens. Certain inulin preparations were effective, but tannic acid proved more effective. Either one in high concentration would agglutinate red cells. Modification of the cell must be carried out with great care for the reaction to occur. The treated cells are exposed to the protein antigen and thus acquire a new serologic specificity in that they are specifically agglutinated in the presence of homologous protein antibodies. Boyden demonstrated hemagglutination of tuberculo-protein modified and streptococcal-protein modified erythrocytes.

Davidsohn and Toharsky (29, 30) reported that Corynebacterium H produced changes in human plasma and serum such that they possessed panagglutinating capacity. Panagglutination, the agglutination of erythrocytes by any normal human serum, was reported by Thomsen in 1927 (80).

Viral hemagglutination. In 1941, Hirst (55) reported the in vitro agglutination of chicken erythrocytes by influenza virus-infected

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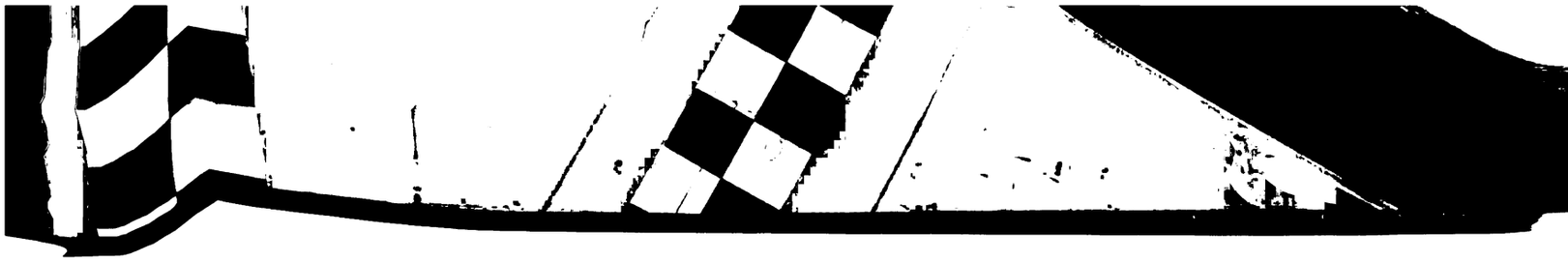


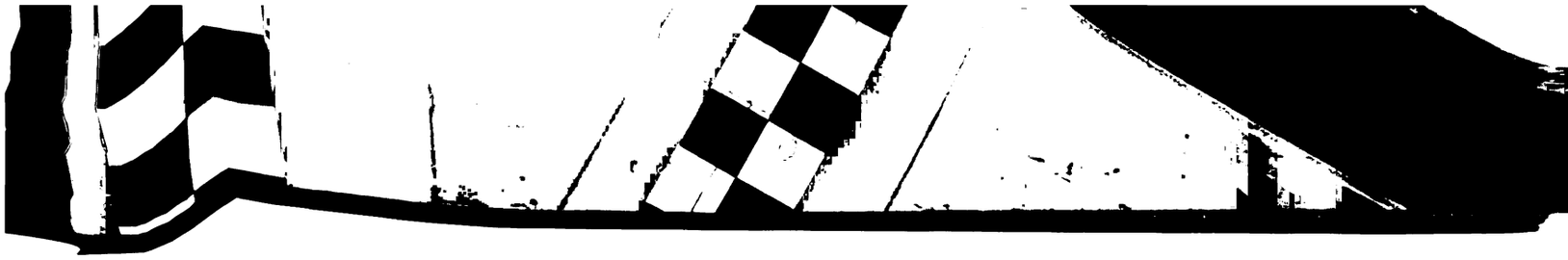
allantoic fluid. Agglutination is characterized by a film of red cells covering the bottom of the tube. Normal allantoic fluid (NAF) in combination with red cells does not produce a pattern different from that of cells settling in physiological saline; i.e., a sharply demarcated disc of cells or "button" formation at the bottom of the tube.

Hirst further demonstrated a strain specific inhibition of hemagglutinative activity by anti-influenza serum.

Influenza virus hemagglutination was also discovered independently by McClelland and Hare in 1941 (72). They reported that human and guinea pig red cells, in addition to chicken erythrocytes, were agglutinable and that the virus was adsorbed by the cells.

The first requirement for the exact study of the phenomenon was the establishment of specific criteria for the determination of hemagglutination titers. Hirst (56) used a method dependent on the optical density of the supernatant fluid. The absorbance of this fluid varies inversely with the rate and degree of agglutination and settling of cells. Salk (85) established the pattern method for reading hemagglutination.

Characteristics of hemagglutination permit separation of viruses into three groups: (1) influenza, mumps, and Newcastle disease; (2) vaccinia, variola, and ectromelia; and (3) a variety of



other viruses--Japanese B, St. Louis and Russian encephalitis, mouse encephalomyelitis (Theiler's GD VII), West Nile fever, encephalomyocarditis group, pneumonia virus of mice, foot-and-mouth disease, and fowl plague.

The mumps (M), Newcastle disease (N), and influenza (I) group (MNI) agglutinates red blood cells of a number of different animals. Chicken, guinea pig, and human cells are more commonly employed. Hemagglutination proceeds in two stages: (1) adsorption of the virus to specific receptors on the cell surface and subsequent agglutination; and (2) elution of the virus from the cells, leaving a modified cell surface (56, 57). The reaction is considered to be an enzymatic process associated with the virus particle.

Separation of the enzymatic activity from the viral particles has not been accomplished, but soluble enzymes of essentially similar quality have been obtained in culture filtrates of a few bacterial species.

Electron microscopy indicates that agglutination is due to the formation of a lattice of red cells held together by multivalent virus particles (53).

Definite evidence for the relationship of cell receptors for the MNI viruses is found in studies of the agglutinability of cells from which a virus has eluted. Elution of M leaves the cells

agglutinable by N or I but not by M. Elution of N renders the red cells agglutinable only by I. Strain differences may be observed with influenza virus. Elution of influenza A leaves cells still agglutinable by some strains of influenza B virus. This provides the basis for the "receptor gradient" theory (13).

The principal investigations of the hemagglutination reaction with the MNI group have utilized influenza virus.

Adsorption of influenza virus to the surface of red cells occurs rapidly in physiological saline. Adsorption can be prevented in a solution of very low ionic concentration (18). In the absence of ions, none of the adsorptive or enzymatic interactions of influenza viruses with mucoid inhibitors or cell receptors occur.

Visible agglutination by active virus may occur in solutions containing as little as 0.01 M monovalent cations. Indicator virus (virus heated at 55-56°C. for 30 minutes which shows no reduction in hemagglutinative activity but which has lost its capacity to elute; i.e., lost its enzymatic activity) reacts in a lower concentration of salts.

Cations are the most important ions in the phenomenon. Calcium ions are more effective than sodium or potassium in allowing adsorption or enzymatic activity. The only anionic effect clearly

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demonstrable is that of sodium hexametaphosphate, which inhibits hemagglutination and probably is due to an anticalcium action.

Adsorption of virus by red cells at 0-4°C. is most effective with 0.1 M salt concentration. Some adsorption occurs at 0.01 M concentration or less, and there is no sharp threshold of demarcation.

At 27°C. destruction of receptors occurs at salt concentration below that required to produce visible agglutination.

The rate of adsorption of influenza virus is related to temperature and numbers of cells (57). An increase of cells is accompanied by an increase in the rate and degree of adsorption of hemagglutinin. The time for maximum adsorption is the same regardless of cell concentration. With the greater concentrations of cells the rate and degree of elution decreases.

At 4°, 27°, and 37°C. adsorption proceeds rapidly during the first minute. Adsorption is less complete with rise in temperature. At 4°C. the maximum adsorption (99.5 per cent of the virus) is not attained until 15 hours; at 27°C. (98.8 per cent), in 25 minutes; and at 37°C. maximum adsorption (87.0 per cent) occurs in three to five minutes.

The amount of elution observed at 4°C. at 18 hours is negligible, but the degree of elution increases with temperature so

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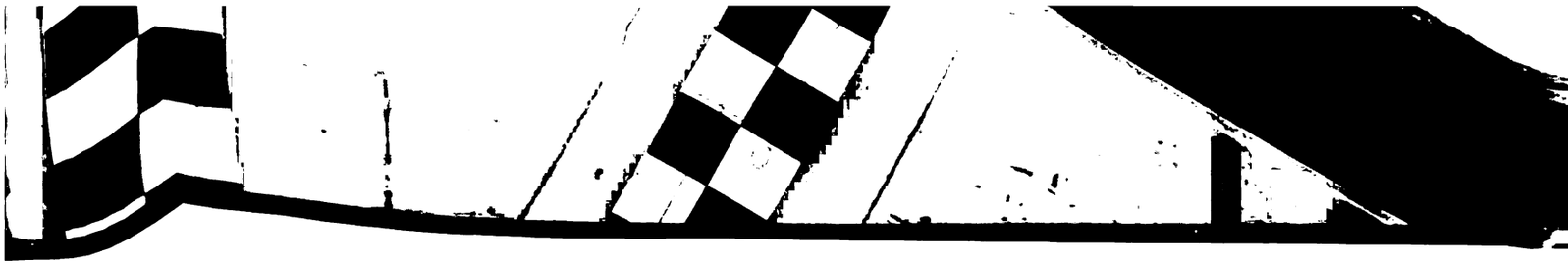


that at 37°C. almost all of the adsorbed agglutinin is released in six hours.

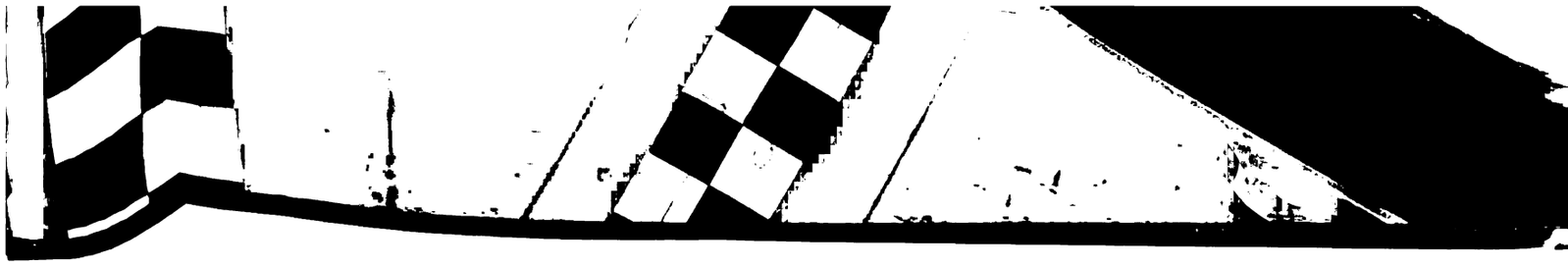
The infective agent of influenza virus suspensions is adsorbed by red cells simultaneously with the adsorption of hemagglutinins. Ninety-five per cent of the infective agent is removed in 15 minutes.

Inhibition of viral hemagglutination. Three serum components are known to inhibit viral hemagglutination: (1) homologous antibody, presumably gamma globulin, specific for the virus antigen; (2) "Francis" inhibitor, active against indicator viruses; and (3) "nonspecific" inhibitor, characterized by its effect on unheated virus.

Francis (44) observed that if indicator virus (LEE) is used to determine the hemagglutination-inhibition (HI) titer of sera, very high values are obtained irrespective of the antibody content.

Anderson (3) demonstrated that the "Francis" inhibitor is destroyed by the receptor-destroying enzyme (RDE) of Vibrio cholerae as well as by active influenza virus. This allowed the development of the hypothesis that influenza virus contained an enzyme essentially similar to the soluble RDE of Vibrio cholerae.

Inhibitory action similar to that of the "Francis" inhibitor is elicited by a variety of physiological secretions and tissue extracts containing mucoprotein. Some of these are human cervical

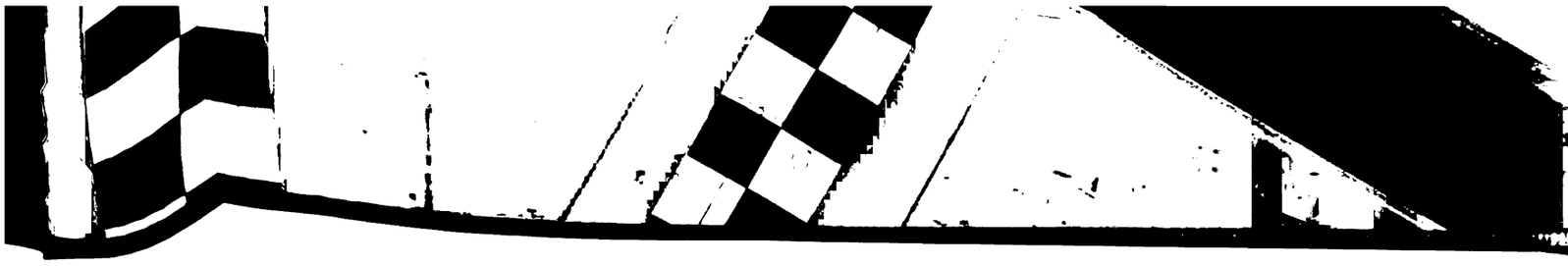


mucus, egg white, tears, human urine, and an extract from the submandibular salivary gland of sheep.

McCrea (75) identified the serum inhibitor of heated LEE virus as a component of the heat-stabile mucoprotein fraction of rabbit and human sera. Based on a dry-weight comparison, purified serum mucoprotein shows considerably higher activity than the serum from which it was derived. Serum mucoid inhibitor is relatively labile in that it is inactivated by heat at pH 4.6, by chloroform at pH 4.6, and by treatment with 90 per cent phenol. Inhibitory activity of normal serum is destroyed by trypsin, chymotrypsin, pepsin, and cyanide-activated papain. All these reactions proceed more readily with increasing purity of the mucoid fraction.

McCrea (73) reported destruction of nonspecific inhibitor in normal rabbit serum in 20 minutes at 62°C.

Friedewald, Miller, and Whatley (45) demonstrated that saline extracts of human and chicken red cells contained inhibitor. These cells could also be agglutinated by influenza or mumps virus. When the virus receptor substance was removed from chicken erythrocytes by adsorption and elution with influenza virus, extracts of the cells no longer yielded the inhibitory substance. The inhibitory substance did not neutralize influenza virus in mice, and it failed to fix complement when mixed with influenza or mumps virus. Evidence was



obtained that some virus was released from the inhibitory substance after incubation for six hours at 22° or 37°C.

Hirst (58) mixed equal portions of normal rabbit serum and crystalline trypsin (250 mg per cent), incubated at 37°C. for three hours, and noted a marked reduction in inhibitory titer using chicken red cells.

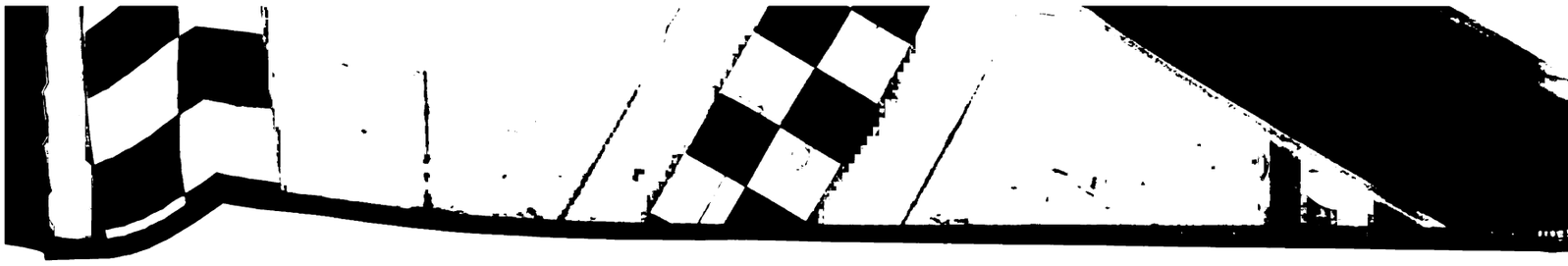
Svedmyr (92) identified the inhibitor of influenza hemagglutination which is present in normal allantoic fluid as a mucoid.

Lanni and Beard (65), working with swine influenza virus, observed that egg white inhibitor is almost completely destroyed by treatment with dilute periodate.

Murphy (78) reported optimal destruction of nonspecific inhibitors of influenza hemagglutination present in serum by the use of RDE at pH 6.0.

Wenners, Monley, and Jenson (95) utilized crystalline trypsin and sodium periodate, respectively, to treat serum and eliminate nonspecific inhibitors of hemagglutination of chicken erythrocytes by mumps and Newcastle disease viruses.

Davoli and Bartolomei-Corsi (31) reduced, and in some instances eliminated, the inhibitory activity of normal rabbit and human sera by adding a 3.81 per cent solution of sodium citrate to the serum.



Sampaio and Isaacs (86) used crystalline trypsin (32 mg per ml of serum at 56°C. for 30 minutes) to destroy inhibitors in normal ferret, chicken, rabbit, guinea pig, and mouse sera.

Tamm and Tyrrell (93) reported that both normal allantoic fluid and human urine inhibit hemagglutination by the murine encephalomyelitis GD VII strain and influenza viruses. The inhibitors of GD VII are not affected by Vibrio cholerae filtrate or by active influenza viruses. The inhibitor contained in normal allantoic fluid combines with GD VII at 4°C. and dissociates from the virus at 22°C. By a combination of chemical purification and starch-zone electrophoresis, it has been shown that the GD VII inhibitor is chemically distinct from the influenza (LEE) virus inhibitor.

Casals (21) reported that nonspecific inhibitors of influenza hemagglutination were removed from 1:5 dilutions of monkey and other animal sera by Seitz filtration. The removal of inhibitors was probably due to adsorption by the filter.

An inhibitor of hemagglutination present in allantoic fluid suspensions of certain strains of Newcastle disease virus, and in normal allantoic fluid of thirteen-day-old embryos, was largely removed by dialysis as reported by Williamson, Simonsen, and Blattner (98). The inhibitor was heat-stabile and was not destroyed by RDE. Red blood cells suspended in allantoic fluids for varying

periods of time regained their agglutinability after washing. The inhibitor retained complete activity.

Cell receptor sites. The nature of red blood cell receptor sites has not been elucidated. After treatment with influenza virus or RDE there is a sharp decrease in the electrophoretic velocity of human erythrocytes, representing a diminution in the net negative charge (1, 50).

Red cells treated with suitable amounts of periodate adsorb viruses of the influenza group, but neither spontaneous elution nor artificial removal by RDE occurs according to Fazekas de St. Groth (41). Erythrocytes treated with periodate-virus RDE (PVR cells) have active virus bound firmly to the modified receptors. PVR cells have the following properties:

1. They do not agglutinate spontaneously.
2. They are not agglutinated by the addition of influenza virus.
3. They agglutinate all cells susceptible to influenza virus agglutinins.
4. They destroy receptors on normal cells and the virus inhibitor in mucoids.
5. They are agglutinated by specific antisera to the virus with which they are incorporated.

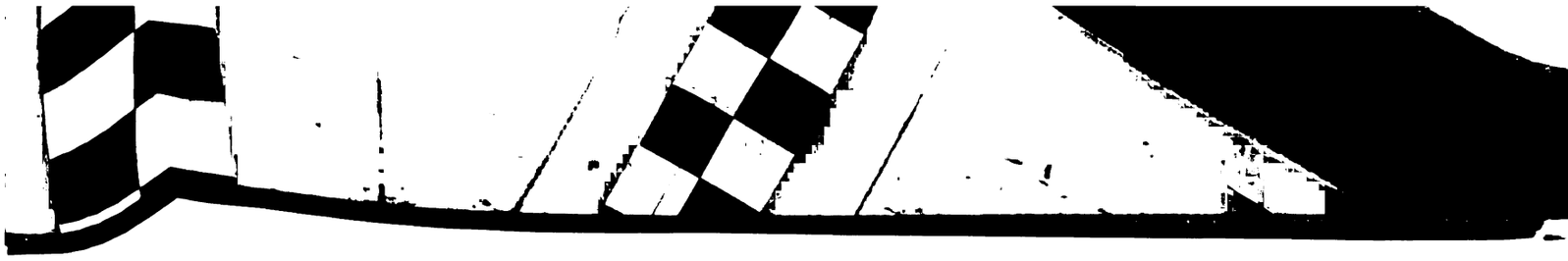
6. The agglutinating and enzymatic activities of these cells are neutralized by homologous antibody.

The influenza virus receptors of chicken red cells resist destruction by a number of oxidizing agents (iodine, hydrogen peroxide, potassium permanganate, and potassium dichromate), but are readily destroyed by sodium periodate at concentrations greater than that required for modification of cellular receptors (58).

Burnet (14) states that the effect of periodate indicates the likelihood that the specific configuration of certain carbohydrate units is responsible for adsorption of virus and for susceptibility to enzymatic action.

Fazekas (42) reported that treatment of influenza virus with relatively large amounts of periodate destroyed infectivity, enzyme activity, antigenicity, and hemagglutinative activity.

Cellular receptors in excised mouse lung and in the formalinized (0.1 ml of 4 per cent formalin) allantoic cavity can be modified so that they adsorb virus but neither spontaneous elution nor artificial liberation by RDE occurs. Adsorbed virus is released from the untreated membranes upon introduction of RDE and also occurs spontaneously. Periodate treatment produces identical receptor modification in vivo, both in mice and eggs (42).

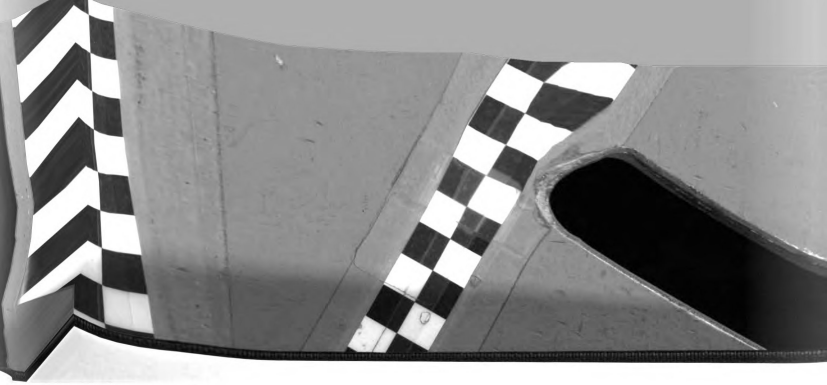


Influenza virus infection may take place through periodate-modified receptors on which RDE cannot act. This may be determined by:

1. Identity of infectivity end points in periodate-treated and in normal mice and eggs.
2. Reduction of the prophylactic effect of RDE in periodate-treated lungs.
3. Absence of interference in the allantoic cavity by heat-inactivated virus in the presence of RDE, if the active virus has been adsorbed to receptors modified by periodate.

Fazekas stated: "The assumption of enzymic receptor destruction as the mechanism of infection (Hirst) is criticized, and a new hypothesis proposed: 'Preceded by specific adsorption of the virus to cellular receptors, infection is initiated by means of viropexis, the passive uptake of the infective particle by the host cell; enzymic destruction of receptors is not essential.'"

Vaccinia and related hemagglutinins. Vaccinia, variola, and ectromelia viruses possess a hemagglutinin that is distinct from the virus particle. Two hemagglutinating fractions have been found in vaccinia virus suspension of infected chorioallantoic membranes.



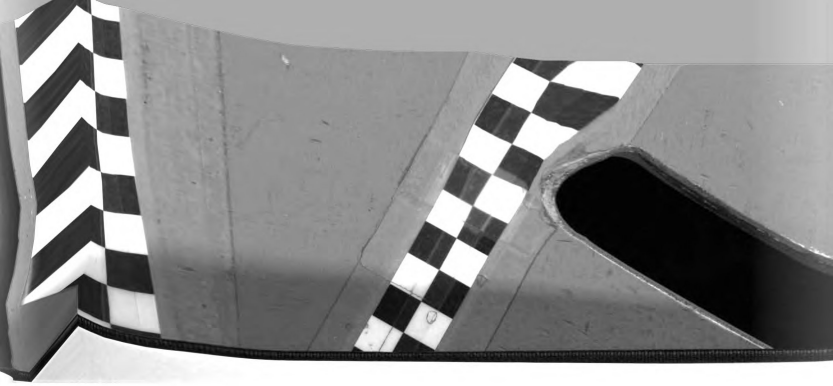
One is a soluble hemagglutinin and is completely separable from the infective virus particle whereas the other is closely associated with the elementary body (16, 46).

Hemagglutinins of this group are inhibited by specific immune sera but not by normal sera. Spontaneous elution of adsorbed hemagglutinin does not occur, but the hemagglutinin can be removed by addition of immune serum and the red cells are reagglutinable.

Vaccinia hemagglutination is demonstrable only with chicken and pigeon cells (90).

Hemagglutination with arthropod-borne viruses (21). The use of acetone and ether extracts of brain tissues of newborn mice infected with certain arthropod-borne viruses has made it possible to demonstrate hemagglutinins for chicken red cells associated with the following viruses: Dengue Type 1, Dengue Type 2, Eastern equine encephalomyelitis (EEE), Ilheus, Japanese B, Ntaya, St. Louis, Sindbis, Uganda S, Venezuelan equine encephalomyelitis (VEE), West Nile (Egypt 101 strain), Western equine encephalomyelitis (WEE), and yellow fever viruses.

On the basis of temperature and pH requirements for hemagglutination, the viruses may be divided into two groups: (1) those that require 37°C. and pH 6.4--EEE, VEE, WEE, and Sindbis viruses;



and (2) those that require either 4°C. or 22°C. and pH 7.0--Dengue Types 1 and 2, Ilheus, Japanese B, Ntaya, St. Louis, Uganda S, West Nile, and yellow fever viruses.

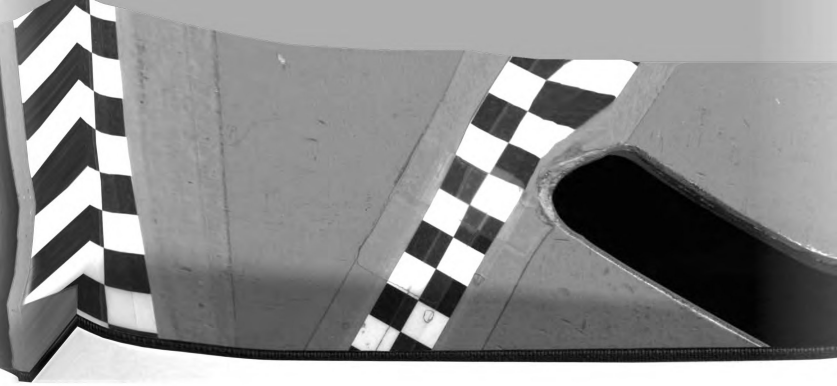
Serological cross reactions may be found among viruses of each group, but antisera to one group do not react with antigens of the other group.

Antibodies against certain viruses for which no hemagglutinin could be detected react with members of either one or the other group. Semliki Forest virus appears to belong to Group 1 and Russian far eastern and louping-ill viruses to Group 2.

Action of enzymes in hemagglutinating systems. Rh-positive erythrocytes treated with trypsin and other proteolytic enzymes (pepsin, papain, erepsin; chymotrypsin, bromelin, and Russel viper venom) are modified so that they show specific agglutination in saline dilutions of incomplete (albumin-active) anti-Rh sera (96).

Agglutination of trypsin-modified cells by incomplete antibodies occurs in the absence of hydrophilic colloids which are required for the agglutination of unmodified cells.

Trypsin, in low concentration, modifies red cells so that they are agglutinated by saline dilutions of homologous antibody. When used in progressively higher concentrations, trypsin alters the red



cells so that they are agglutinated first by most normal sera and finally by saline.

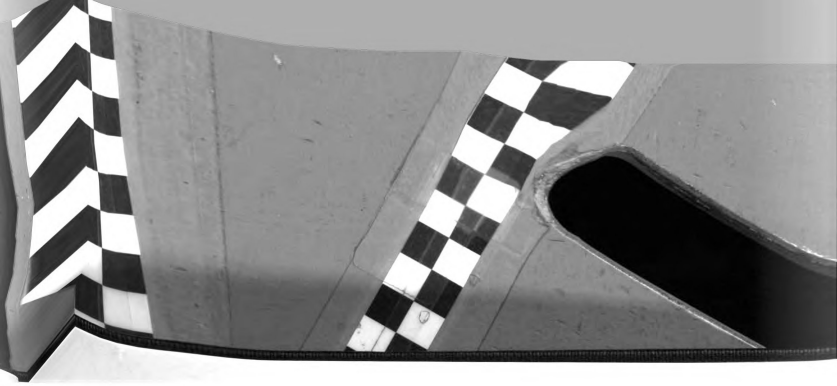
Agglutination of the more vigorously treated red cells by normal sera may be due to the T agglutinin found in many normal sera. The autoagglutination by saline of the most vigorously trypsin-treated erythrocytes, however, is a nonspecific effect (97).

Morris (77) treated mouse brain suspensions of the GD VII strain of murine encephalomyelitis virus with trypsin which induced the viral agglutination of human group O cells at 20°C., whereas untreated virus agglutinated these cells only a 4°C.

An effect on the red cell was excluded because washed, trypsin-treated cells failed to agglutinate at 20°C. in the presence of the untreated virus. Trypsin-treated virus, sedimented and washed by high-speed centrifugation, agglutinated human cells well at 20°C.

Enzymes and Enzyme-inhibitors

The major proteolytic enzymes of pancreatic juice are trypsin, chymotrypsin, and a carboxypolypeptidase, each of which has been obtained in pure form. The combined activities of these enzymes was formerly believed to be due to trypsin alone. The enzyme combination is referred to as pancreatin (52).



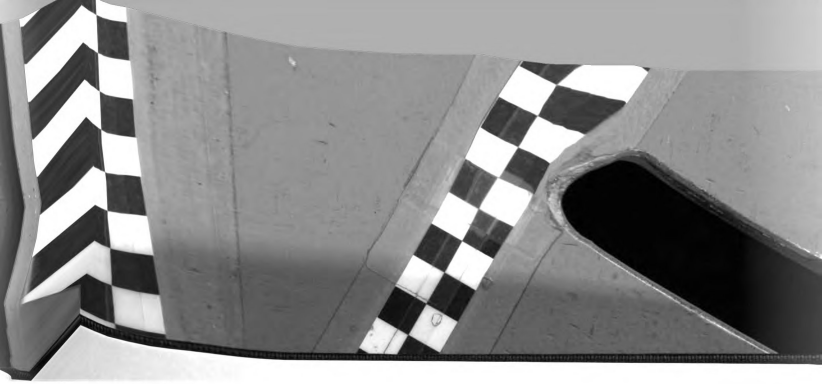
Trypsin and chymotrypsin may be classified as endopeptidases acting on peptide linkages either in the central or terminal portions of polypeptide chains. Bergmann and Fruton (8), through the use of synthetic peptide substrates, found that trypsin acts on peptide linkages containing the carboxyl groups of either arginine or lysine. Chymotrypsin, on the other hand, was found to act on peptide linkages involving the carboxyl group of tryosine and phenylalanine. The digestive action of trypsin and chymotrypsin appears to involve the splitting of specific types of peptide linkages in the molecule, thereby producing either low molecular weight polypeptides or free amino acids.

Trypsin has its greatest activity at pH 8 to 9. The optimum pH depends on ionic strength of the buffer, concentration of the substrate, and somewhat on the nature of the substrate.

Carboxypolypeptidase activity is most likely due to a complex of many enzymes and is an example of an exopeptidase.

In addition to the principal enzymes, pancreatic amylase and pancreatic lipase are found in the pancreatic juice.

Activation of the major proteolytic enzymes of the pancreas is presented in the following:

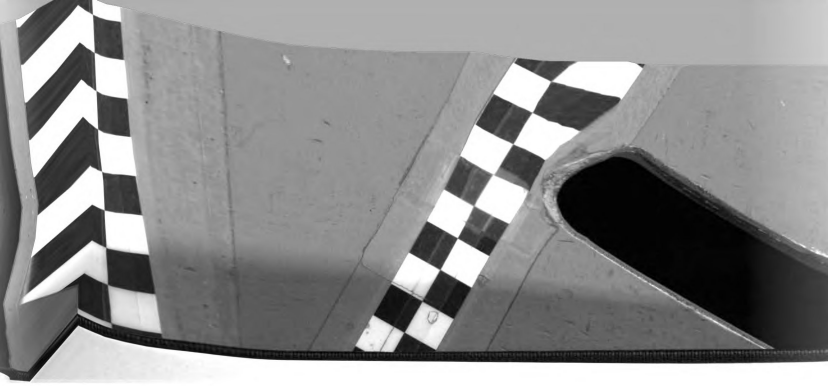


Precursor	Activator	Enzyme
Trypsinogen	Neutral pH or Enterokinase	Trypsin
Chymotrypsinogen	Trypsin	Chymotrypsin
Procarboxypolypeptidase	Trypsin	Carboxypolypeptidase

The peptide bonds are not the only groups attacked by the enzymes, since hydrolytic cleavage also occurs if the peptide bond is replaced by an ester bond.

The mode and extent of cleavage are not completely delineated. Large amounts of free amino acids are formed when crude preparations of pancreatin act on casein or other proteins, but no amino acids are formed when casein is exposed to the action of crystalline trypsin (51).

There are a number of naturally occurring trypsin inhibitors such as the soybean, colostrum, lima bean, ovomucoid, blood plasma, urine, Ascaris sp., and pancreatic inhibitors (66). Increased inhibition of trypsin by serum is associated with disease processes that bring about cellular destruction. The diagnostic significance of increased trypsin or chymotrypsin inhibition is the same as that of increased fibrinogen concentration. It is a common, nonspecific response to a variety of pathological conditions, and has no value as a specific diagnostic test (88).



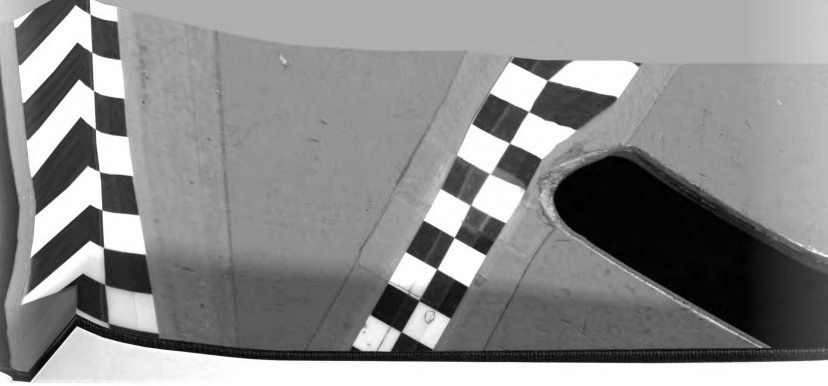
Gorini and Audrain (47) reported that after addition of one equivalent of ovomucoid to one equivalent of trypsin about 7 per cent of the tryptic activity remained. They ascribed this remaining activity to the enzyme-inhibitor complex itself. The addition of calcium to the system resulted in a gradual disappearance of ovomucoid. They concluded that the simultaneous presence of calcium and trypsin inactivated the ovomucoid and rendered it susceptible to proteolytic digestion. Inactivation did not occur in the absence of trypsin and occurred very slowly in calcium-free systems.

Later (48), they concluded that the ovomucoid complex behaved as an enzyme with a proteolytic activity corresponding to about 10 per cent of the activity of the trypsin present.

Only traces of digestion of soybean inhibitor are noticed after prolonged incubation in the presence of trypsin (64).

Lima bean inhibitor exerts its full action immediately upon contact with trypsin. The amount of trypsin inhibited is directly proportional to the amount of inhibitor present, indicating a stoichiometric reaction (68).

Green (49) states that the inhibition of trypsin by diisopropyl fluorophosphate, ovomucoid, soybean, and pancreatic inhibitors is of the competitive type, since it is overcome in the presence of the substrate, benzoyl-L-arginine ethyl ester.



EXPERIMENTAL PROCEDURES

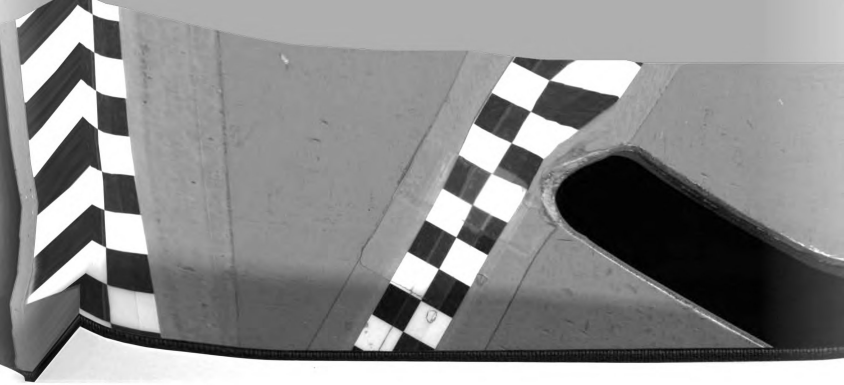
The study was conducted in two parts: (1) the investigation of hemagglutinative activity associated with IBV; (2) the possible use of IBV hemagglutinative activity for diagnosis and characterization of IB.

Materials and Preparations

1. Viruses:

The strains of IBV used were from the North Central IBV Repository at Michigan State University, and are listed below:

Repository Code No.	Code	Isolated by	Egg Passage
1	314	M. S. Hofstad, Iowa State College, Ames, Iowa (1948)	6
2	33	M. S. Hofstad, Cornell University, Ithaca, New York (1944)	9
3	104	M. S. Hofstad, Iowa State College, Ames, Iowa (1947)	4
16	66	O. Hipolito, Universidade Rural de Estade de Minas Gerais, Escola Superior de Veterinaria, Brazil (1955)	30
19	999	C. S. Roberts, Alabama Dept. Agr. (1956)	4

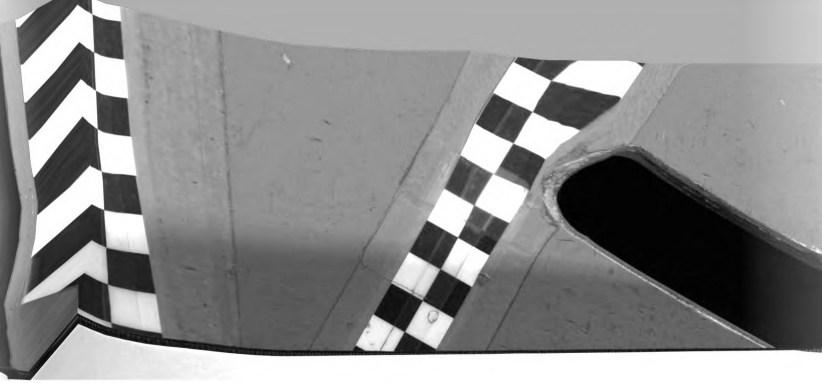


Repository Code No.	Code	Isolated by	Egg Passage
20	603	C. S. Roberts, Alabama Dept. Agr. (1956)	9
21	571	C. S. Roberts, Alabama Dept. Agr. (1956)	6
23	331	C. S. Roberts, Alabama Dept. Agr. (1956)	13
24	SIMS	C. S. Roberts, Alabama Dept. Agr. (1956)	9
40	586	M. P. Spring, Michigan State U. (1956)	0
41		H. Van Roekel, U. of Massachusetts (1941) (291st bird passage)	0
42		F. R. Beaudette, Rutgers U., New Brunswick, New Jersey (1935)	Unknown (hundreds)

The strains of IBV will be identified by the Repository code number and number of egg passages; e.g., 40-5 indicates repository code 40, fifth egg passage.

IBV strain 42 is completely egg-adapted. This strain has been studied extensively and is often referred to in the literature as the Beaudette strain.

A strain of Newcastle disease virus (NDV) (Accession 51-52-308) which originally had been isolated at Michigan State University from lung and tracheal material of chickens infected from a natural outbreak of the disease was used for certain comparative studies.



Normal allantoic fluid served as a control.

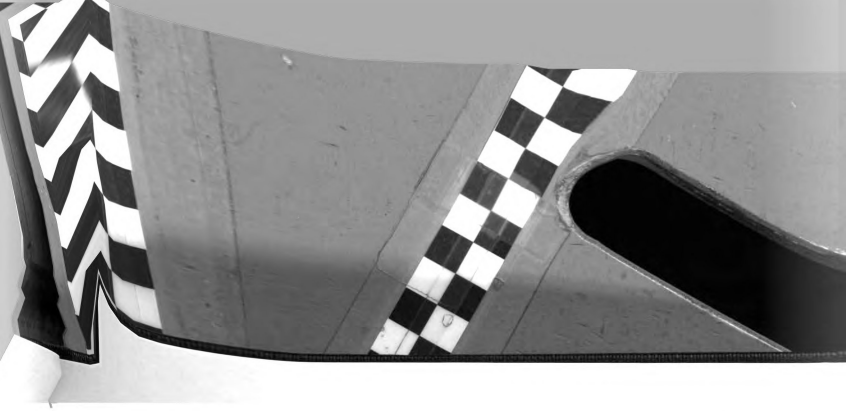
All viruses were cultivated in embryonating chicken eggs via the allantoic cavity (0.1 ml inoculum). Nine-day embryos were used for all strains of IBV except the egg-adapted strain, which was inoculated into ten-day embryos as was NDV. All normal and viral-infected allantoic fluids were collected from twelve-day embryos.

At the time of harvest each allantoic fluid preparation was pooled, distributed in 30 ml screw-cap vials, and stored at -30°C. until used.

At the time of use the virus suspension was thawed, centrifuged at 3,000 r.p.m. for 20 minutes at 4°C., and the clear supernatant fluid was removed for the tests. Samples were frozen only once as repeated cycles of freezing and thawing have a deleterious effect on IBV infectivity.

2. Erythrocytes:

Four adult Single Comb White Leghorns served as the source for erythrocytes which were obtained by cardiac puncture. Blood was collected in chemically clean test tubes containing 1 ml of a 2 per cent sodium citrate solution for each 9 ml of blood. The cells were washed three times in ten or more parts of 0.85 per cent saline, centrifuged twice at 1,600 r.p.m. for eight minutes, and



finally at 1,000 r.p.m. for 10 minutes. After the third washing the saline was removed and the packed cells were stored at 4°C. Cells were used in hemagglutination tests within one week of the time of collection.

The author was the donor of human O cells.

Cow, horse, sheep, dog, cat, and rabbit erythrocytes were collected from animals available in the College of Veterinary Medicine.

3. Diluents:

0.85 per cent sodium chloride.

Bacto hemagglutination buffer (Difco), pH 7.2.

4. Sera:

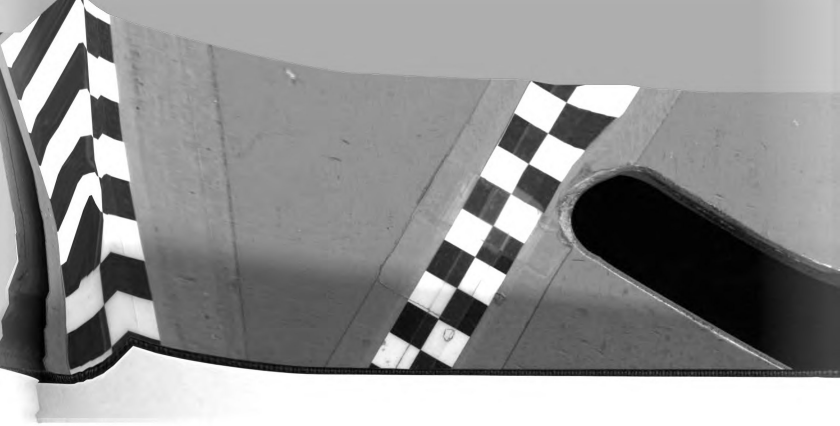
Normal serum was collected from chickens reared in isolation. Anti-IB serum was collected six weeks after intratracheal inoculation of adult chickens with 0.5 ml of IBV. All sera were quantitatively assayed by serum-neutralization tests.

5. Enzymes:

Trypsin, 1:250 (Difco).

Trypsin (1 per cent) for hemagglutination (Difco).

Trypsin, 2× crystalline, salt-free (Nutritional Biochemical Corporation--NBC).



Chymotrypsin, salt-free, from ethyl alcohol (NBC).

Chymotrypsinogen, crystalline, salt-free (NBC).

Papain, N.F. (Difco).

Pepsin, 1:10,000 (Difco).

Rennin, N.F. (Difco).

6. Enzyme inhibitors:

Eggwhite trypsin inhibitor (NBC).

Soybean trypsin inhibitor, 5× crystalline (NBC).

7. Amino acids and peptones:

L-arginine, free base (NBC).

Glycine, anhydride (NBC).

L-lysine, monohydrochloride (NBC).

L-methionine (NBC).

L-phenylalanine (NBC).

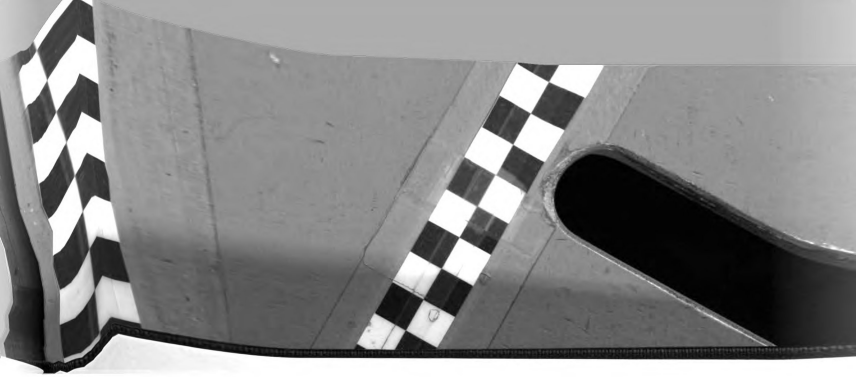
L-serine (NBC).

L-tyrosine (NBC).

Trypticase (Baltimore Biological Laboratories).

Bacto-Tryptone (Difco).

Bacto-Tryptose (Difco).



8. Bacterial filtrates:

Clostridium welchii, types A, C, and D, were incubated at 37°C. for 18 hours in a medium of equal parts of pork infusion and thioglycollate broth. The cultures were centrifuged at 3,200 r.p.m. for 30 minutes at 4°C. and the supernatant fluid was collected.

9. Colloids:

Arsenic trioxide.

Red colloidal gold.

Chromium hydroxide.

Ferric hydroxide.

Prussian blue.

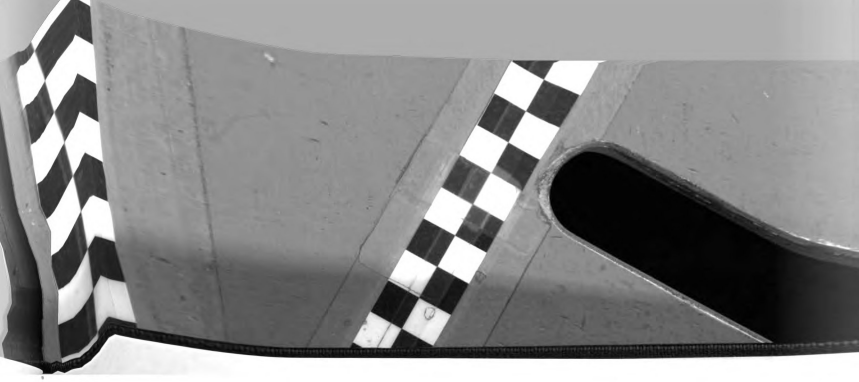
Silver.

Methods

1. Hemagglutination (HA) test:

Serial twofold dilutions of the virus samples were prepared with Bacto hemagglutination buffer through the range of 1:5 to 1:2560 or higher, as required.

In a row of 12 × 75 mm tubes parallel to the tubes containing the virus dilutions, 0.25 ml each of the buffer, appropriate virus dilution, and 0.5 per cent suspension of chicken red cells were added



to each tube. In some tests cells from other species were used. The control tube contained 0.5 ml of buffer and 0.25 ml of cells.

The tubes were shaken well and incubated for one hour at room temperature. In some instances they were incubated at different temperatures.

The end point was the greatest dilution of virus which showed a pattern of complete hemagglutination. No attempt was made to read partial reactions.

The titer, expressed as hemagglutinative units, was the reciprocal of the highest dilution of virus, before the addition of saline and cells, which produced complete hemagglutination.

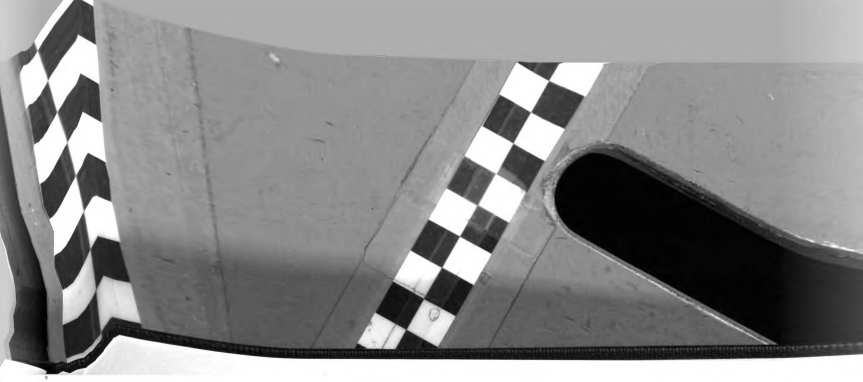
2. Hemagglutination-inhibition (HI) test:

a. 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 in each tube. 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:



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

b. Beta procedure:

Constant amounts of virus, expressed in HA units, were employed with decreasing concentration of serum ranging from 1:5 through 1:2560.

The serum titer is the reciprocal of the highest dilution of the serum in which hemagglutination is completely inhibited. The HI titer of the serum is computed for the beta procedure by multiplying the serum titer by the number of HA units used in the test. The following formula is used:

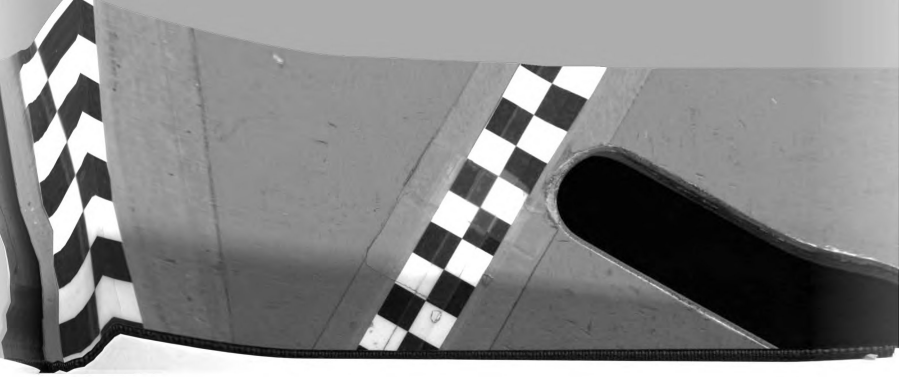
Serum titer × number of HA units = HI titer.

3. Hemagglutination test using trypsin-modified IBV:

The HA test developed during the present study and used for investigation of characteristics of IBV employed the same procedure as the conventional HA test. The only difference was that IBV modified by trypsin was used. The modified IBV was also used for the HI test.

4. Viral infectivity:

For quantitative determination of viral infectivity, serial tenfold dilutions of the virus were prepared in nutrient broth 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. in an electric forced-draft incubator for five days following inoculation. Embryo mortality during the first 24 hours was considered to be due to nonspecific causes, and these were not included in the final results.

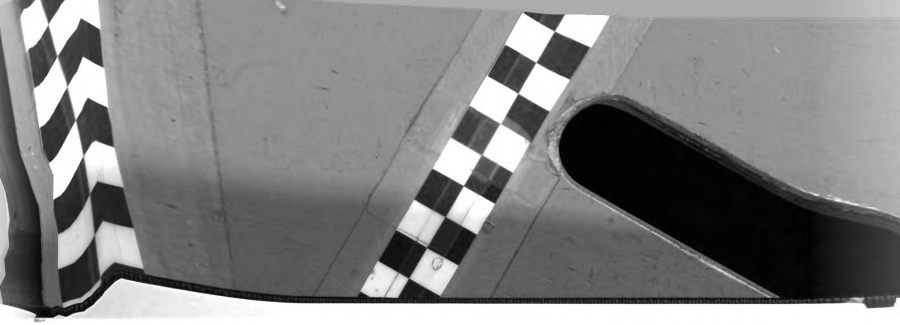
Mortality and gross lesions were the criteria for infectivity of strains other than the egg-adapted strain 42, and were used in computing the titer, which was expressed as the 50 per cent infectivity end point, ID_{50}, according to the method of Reed and Muench (84). Lethality was the criterion for strain 42, and the titer was expressed as the LD_{50}.

5. Serum-neutralization tests:

Dilutions were prepared in the same manner as described for infectivity titration. Two rows of tubes were set up parallel to the dilution tubes. One series of tubes contained 0.3 ml of a 1:5 dilution of the test serum per tube. The second series contained 0.3 ml of nutrient broth per tube. To each was added 0.3 ml of the corresponding virus dilution. Separate pipettes were used for preparing all mixtures. Inoculation was via the allantoic cavity, 0.1 ml per egg, five eggs per dilution, and incubation and

observations were made as described earlier. The series containing serum was inoculated prior to the controls to exclude the possibility of thermal environmental effects on the virus.

The 50 per cent end point was used to evaluate all titrations. The LD_{50} neutralization index ($LD_{50}NI$) was the difference between the reciprocal of the virus and the serum titers. The antilog was the number of neutralizing doses.



EXPERIMENTAL RESULTS

Hemagglutination

pH of normal and viral-infected allantoic fluid. Since the investigation would utilize trypsin and perhaps other proteolytic enzymes, it was necessary to determine the pH of normal and viral-infected allantoic fluid to ascertain this environmental factor which influences enzyme activity. The results are presented in Table 1. The pH range of the samples tested was within the optimum, pH 8-9, for the activity of trypsin (79).

Modification of infectious bronchitis virus by trypsin. The initial approach to modification of IBV by trypsin was concerned with the concentration of trypsin and the effects of time and temperature on the reaction.

A 1 per cent aqueous suspension of trypsin (T) (1:250, Difco) was prepared and passed through a Seitz EK filter pad. Although T is not completely soluble in water at this concentration, reference will be made to the filtrate as 1 per cent T. Appropriate volumes of 1 per cent T were added to the allantoic fluid preparations to give the desired final concentration of T; e.g., 1 ml of

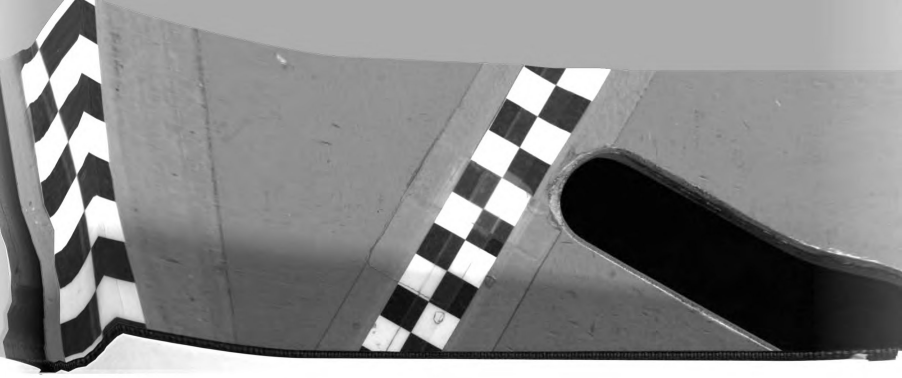


TABLE 1

pH OF NORMAL AND VIRAL-INFECTED ALLANTOIC FLUID FROM TWELVE-DAY-OLD CHICKEN EMBRYOS

Sample	Age of Embryo When Inoculated	pH
NAF[a]		8.49
IBV 40-5[b]	9 days	8.02
IBV 42	10 days	8.09
NDV[c]	10 days	8.52

[a]NAF: Normal allantoic fluid.

[b]IBV: Infectious bronchitis virus with respiratory code number.

[c]NDV: Newcastle disease virus.

1 per cent T was added to 2 ml of allantoic fluid to give a final concentration of 0.33 per cent T.

The 1 per cent T solution, alone or in combination with saline, served as the control.

The results of these studies are presented in Tables 2, 3, and 4.

Hemagglutination occurred with the controls as well as with the normal and viral-infected specimens. It was evident that higher

TABLE 2

HEMAGGLUTINATION BY TRYPSIN-MODIFIED IBV

Strain (repository number)	Final Concentration of Trypsin (pct.)	Temperature (°C.)	Time (min.)	HA Titer	HA Titer after Addition of EWTI
40-6	0.5	4	150	320	. . . .
40-6	0.5	23	120	1280	. . . .
40-5	0.5	37	45	640	. . . .
40-6	0.5	37	90	1280	. . . .
Saline control	0.25	23	0	1280	0
Saline control	0.25	37	180	1280	0
40-6	0.25	4	225	640	. . . .
40-6	0.25	4	270	320	. . . .
40-6	0.25	23	120	640	. . . .
40-8	0.25	23	120	640	. . . .
40-6	0.25	23	180	1280	. . . .
40-6	0.25	23	180	1280	. . . .
40-6	0.1	23	240	20	. . . .
40-6	0.1	23	720	320	. . . .
40-6	0.1	37	180	160	. . . .
40-6	0.1	37	375	640	. . . .
40-6	0.33	23	180	640	. . . .
40-6	0.33	23	240	640	. . . .
40-6	0.33	23	720	2560	. . . .
40-8	0.33	37	120	640	. . . .
40-6	0.33	37	375	2560	. . . .
40-8	0.33	37	180	2560	. . . .

TABLE 2 (Continued)

Strain (repository number)	Final Concentration of Trypsin (pct.)	Temperature (°C.)	Time (min.)	HA Titer	HA Titer after Addition of EWTI
40-6	0.33	37	180	2560	....
40-5	0.33	37	180	5120	....
40-4	0.33	37	180	5120	....
40-5	0.33	37	180	640	320
40-5	0.33	37	180	5120	2560
40-5	0.33	37	180	....	1280
40-5	0.33	37	180	....	5120
40-5	0.33	37	180	....	5120
40-5	0.33	37	180	....	10240
40 5	0.33	37	180	....	10240
40-5	0.33	37	180	....	10240
40-6	0.33	37	180	....	2560
40-15	0.33	37	180	5120	2560
40-16	0.33	37	180	5120	2560
40-17	0.33	37	180	5120	2560
1-7	0.33	37	180	....	2560
2-10	0.33	37	180	....	5120
23-14	0.33	37	180	....	2560
40-3	0.33	37	180	....	5120
40-25	0.33	37	180	....	1280
Saline control	0.33	37	180	1280	0

TABLE 3

HEMAGGLUTINATION BY TRYPSIN-MODIFIED IBV
(egg-adapted strain 42; incubation at 37°C. for 180 minutes; final concentration of trypsin, 0.33 per cent)

HA Titer	HA Titer after Addition of EWTI
640	. . .
640	. . .
640	. . .
640	. . .
1280	. . .
1280	. . .
2560	. . .
2560	. . .
2560	. . .
. . . .	0
. . . .	0
. . . .	0
160	0
320	0
320	0
320	0
640	0
640	0
1280	0
1280	10
2560	0
2560	0
2560	20
2560	160
2560	320
2560	320

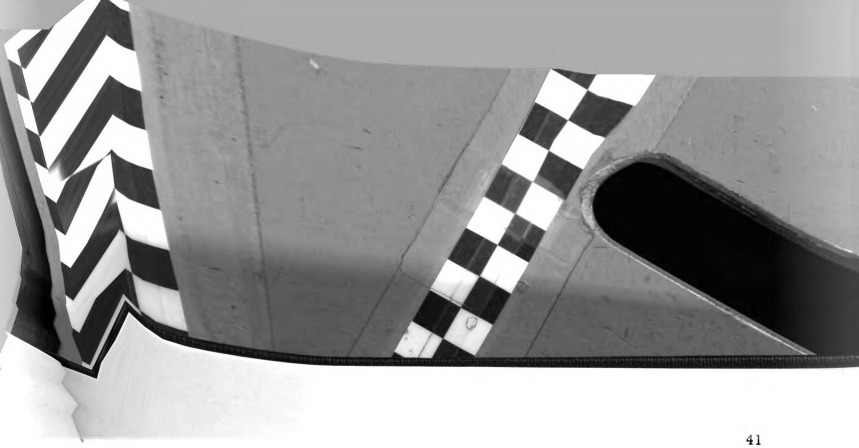
TABLE 4

HEMAGGLUTINATION BY TRYPSIN-MODIFIED NORMAL ALLANTOIC FLUID

Final Concentration of Trypsin (pct.)	Temperature (°C.)	Time (min.)	HA Titer	HA Titer after Addition of EWTI
0.5	4	150	320	. . .
0.5	23	120	160	. . .
0.5	37	45	320	. . .
0.5	37	90	320	. . .
0.25	4	225	40	. . .
0.25	4	270	20	. . .
0.25	23	120	40	. . .
0.25	23	180	20	. . .
0.25	23	180	80	. . .
0.1	23	240	40	. . .
0.1	23	720	160	. . .
0.1	37	180	10	. . .
0.1	37	375	160	. . .
0.33	23	240	640	. . .
0.33	23	720	160	. . .
0.33	37	120	40	. . .
0.33	37	240	640	. . .
0.33	37	375	320	. . .
0.33	37	180	40	. . .
0.33	37	180	160	. . .
0.33	37	180	160	. . .

TABLE 4 (Continued)

Final Concentration of Trypsin (pct.)	Temperature (°C.)	Time (min.)	HA Titer	HA Titer after Addition of EWTI
0.33	37	180	640	. . .
0.33	37	180	. . .	0
0.33	37	180	. . .	0
0.33	37	180	. . .	0
0.33	37	180	10	0
0.33	37	180	10	0
0.33	37	180	20	0
0.33	37	180	40	0
0.33	37	180	160	0
0.33	37	180	320	0
0.33	37	180	320	0
0.33	37	180	320	0
0.33	37	180	640	0
0.33	37	180	640	10
0.33	37	180	640	0



HA titers were obtained with IBV preparations than with the controls or NAF. Although the results were somewhat variable, the highest and most uniform HA titers were obtained with samples containing a final concentration of 0.33 per cent T and which had been incubated for three hours at 37°C. These conditions were selected for the basic procedure.

To determine if the reaction was due to the virus or to the T alone, inhibition of T was attempted. Inhibition of T in the T-IBV mixtures at 100°C. for two minutes caused macroscopic precipitation of proteins which made the suspension valueless for the HA test. The supernatant fluid did not possess hemagglutinative activity.

Resort was then made to naturally occurring T inhibitors. Eggwhite trypsin inhibitor (EWTI) was initially selected because of economy and solubility.

Soybean trypsin inhibitor (SBTI) (5× crystalline) was used in the latter part of the study for comparison to EWTI. The T-SBTI complex exhibits no tryptic activity, and SBTI is resistant to tryptic digestion. EWTI is slowly digested by T and the T-EWTI complex may exhibit as much as 10 per cent of the digestive activity of T alone (47, 64).

Following incubation of the samples with T, inhibitor was added and the HA test performed. A 1 per cent solution of EWTI

was used. The final concentration of EWTI in the mixture was equal to the T concentration. For example, 1 ml of 1 per cent T plus 2 ml of sample resulted in a concentration of 0.33 per cent T. Addition of 1 ml of 1 per cent EWTI to this mixture resulted in a final concentration of 0.25 per cent T and EWTI, respectively. This provides equal amounts of T and EWTI on the basis of weight by volume per cent, but not molality. Different volumes of T and EWTI may be used provided the final concentrations are equal. The molecular weight of EWTI is 28,000 as determined by osmotic pressure and 32,000 as determine by sedimentation diffusion. The average molecular weight of trypsin is 24,000 (66).

Due to the relative insolubility of SBTI, a 0.25 per cent suspension was used. To insure inhibition of T by an equal volume of SBTI, a 0.25 per cent solution of T was necessary.

The results obtained with EWTI are presented in Tables 2, 3, and 4.

The final mixture in the HA test was at pH 7.2, which is within the range (pH 6 to pH 8) of stability of the influenza hemagglutinin. Chicken red cells are spontaneously agglutinated or hemolyzed at greater extremes of pH (61).

It is evident that hemagglutination is associated with the virus modified by treatment with trypsin. EWTI eliminated

hemagglutination by T in the saline controls and NAF. It is obvious that the hemagglutinative activity of the egg-adapted strain of IBV (IBV 42) was considerably less than that of other strains. SBTI, used in parallel with EWTI, provided identical titers with the strains tested.

Based on these findings the viral modification procedure was standardized. The standard reaction system comprised 1 ml of 1 per cent T in combination with 2 ml of normal or viral-infected allantoic fluid. This mixture was incubated at 37°C. for three hours and 1 ml of 1 per cent EWTI was then added. The final mixture was tested for hemagglutinative activity. This procedure was considered to be the "Standard T Process."

The system was maintained bacteria free until the addition of EWTI. Filtration resulted in diminished activity of EWTI. The diminished activity was a reflection of reduced concentration which likely resulted from loss of EWTI by adsorption to the filter.

Repository strain IBV 40-5 was selected as the standard test strain.

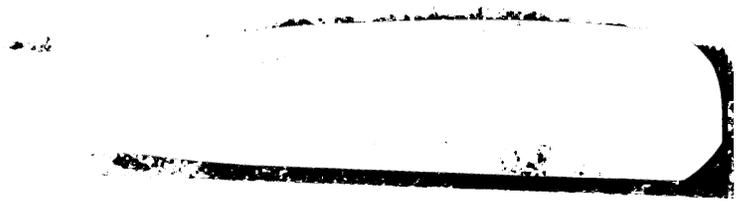
Further results, representative of the standard T process, are presented in Table 5.

A temperature control was run in order to determine the effect of incubation at 37°C. on the virus and its role in the

TABLE 5

HEMAGGLUTINATION BY TRYPSIN-MODIFIED IBV AND NAF (STANDARD T PROCESS)

Saline Control	IBV 40-5	IBV 42	NAF
0	640	20	0
0	1280	0	0
0	2560	20	0
0	2560	0	0
0	2560	0	0
0	2560	0	0
0	2560	0	0
0	2560	0	0
0	2560	0	0
0	2560	0	0
0	2560	0	0
0	5120	320	0
0	5120	0	0
0	5120	20	0
0	20480	0	0
0	20480	40	0



induction of the hemagglutinin. NDV was introduced as a positive control for hemagglutination. The results are presented in Table 6.

TABLE 6

EFFECT OF INCUBATION AT 37°C. ON HEMAGGLUTINATION TITER

Strain	Period of Incubation			
	None	One Hour	Two Hours	Three Hours
NDV	1280	1280	1280	1280
IBV 40-5	0	0	0	0
IBV 42	0	0	0	0
NAF	0	0	0	0

Incubation for extended periods at 4°, 23°, or 37°C., in itself, confers no hemagglutinative activity to IBV (27).

To exclude the possibility of a nonspecific reaction, further studies on the tryptic activity were done, and NDV was included in the series. The effect of varied periods of incubation was concurrently determined. The results are expressed in Table 7.

The data further establish the optimum time of incubation as three hours.

TABLE 7

EFFECT OF TIME OF INCUBATION AT 37°C. ON HEMAGGLUTINATION IN STANDARD T PROCESS

Time of Incubation	IBV 40-5	IBV 40-5	IBV 40-5	NDV
None	20	40	20	320
1 hour	80	1280	640	320
2 hours	160	5120	2560	320
2-1/2 hours	320	....	...	320
3 hours	320	10240	5120	320
4 hours	160	....	...	...

Modification of infectious bronchitis virus by other proteolytic enzymes. Other proteolytic enzymes tested to determine their activity in this system were:

1. Pepsin, 1 per cent solution.

2. Rennin, 1 per cent solution.

3. Papain, 0.5 per cent solution.

The enzymes were prepared in distilled water, passed through Seitz filter pads, and employed in the following manner:

One milliliter of enzyme solution was added to 2 ml of the normal and viral-infected allantoic fluids and incubated at 37°C. for

three hours. No inhibitors were added. The enzyme-fluid mixtures were then used in HA tests. The results are presented in Table 8.

TABLE 8

EFFECT OF CERTAIN ENZYMES ON HEMAGGLUTINATION
(incubation for three hours at 37°C.)

Enzyme	IBV 40-5	IBV 42	NAF	NDV
None	0	0	0	320
Trypsin	5120	1280	160	320
Pepsin	0	0	0	320
Rennin	0	0	0	320
Papain	0	0	0	320

Of the four enzymes employed, only trypsin was active on IBV at pH 8-9 as evidenced by HA. NDV was used as a control, and its hemagglutinative activity was in no way changed by the action of the enzymes.

A further investigation of enzyme activity was undertaken through the use of crystalline trypsin (2×T), chymotrypsin, and chymotrypsinogen. Since no inhibitor for chymotrypsin was available, chymotrypsinogen was used with 2×T, its activator, and

inhibition of chymotrypsin in this system was controlled by inactivating the 2×T with EWTI.

Different volumes and concentrations of crystalline enzymes were employed in these tests. This was predicated on the factor of differential solubility of the crystalline enzymes as compared to T. Purity of crystalline enzymes as compared to T had also to be considered.

The activity of the crystalline enzymes is presented in Tables 9, 10, and 11.

It is evident from the results that 2×T is more reactive than T in this system. One per cent 2×T cannot be substituted for 1 per cent T in the standard T process, whereas 0.25 per cent 2×T may be used. This discrepancy resulted from the differential solubilities of T and 2×T. The 2×T is completely soluble in water at a concentration of 1 per cent, whereas the maximum solubility of T is approximately at a concentration of 0.25 per cent. Thus, 1 per cent 2×T was more reactive and the resultant viral product had no capacity for hemagglutination.

At the optimum 2×T concentration, HA was evidenced in high titer with IBV, in reduced titer with the egg-adapted strain, IBV 42, and not at all with NAF.

TABLE 9

EFFECT OF CRYSTALLINE TRYPSIN ON HEMAGGLUTINATION

Trypsin	Sample	Incubation at 37°C.	EWTI 1 pct.	HA
1 ml 1%	2 ml 40-5	1 hour	1 ml	0
1 ml 1%	2 ml 40-5	2 hours	1 ml	0
1 ml 1%	2 ml 40-5	3 hours	1 ml	0
1 ml 0.16%	2 ml 40-5	1 hour	1 ml	0
1 ml 0.16%	2 ml 40-5	2 hours	1 ml	0
1 ml 0.16%	2 ml 40-5	3 hours	1 ml	0
1 ml 0.33%	2 ml 40-5	1 hour	1 ml	0
1 ml 0.33%	2 ml 40-5	2 hours	1 ml	0
1 ml 0.33%	2 ml 40-5	3 hours	1 ml	0
1 ml 0.75%	2 ml 40-5	1 hour	1 ml	0
1 ml 0.75%	2 ml 40-5	2 hours	1 ml	0
1 ml 0.75%	2 ml 40-5	3 hours	1 ml	0
0.25 ml 1%	2 ml 40-5	3 hours	0.25 ml	1280
0.125 ml 1%	2 ml 40-5	3 hours	0.25 ml	2560
0.25 ml 1%	2 ml 40-5	3 hours	0.25 ml	5120
0.25 ml 1%	2 ml 40-5	3 hours	0.25 ml	5120
0.25 ml 1%	2 ml 42	3 hours	0.25 ml	160
0.25 ml 1%	2 ml NAF	3 hours	0.25 ml	0
1 ml 1%	.....	.....	.....	2560
1 ml 1%	.....	.....	1 ml	0

TABLE 10

EFFECT OF CHYMOTRYPSIN ON HEMAGGLUTINATION
(incubation for three hours at 37°C.)

Chymotrypsin	Sample	HA
1 ml 1%	2 ml 40-5	640
1 ml 1%	2 ml 42	80
1 ml 1%	2 ml NAF	0
0.25 ml 1%	2 ml 40-5	640
0.25 ml 1%	2 ml 42	40
0.25 ml 1%	2 ml NAF	0
1 ml 1%	. . . . .	640

TABLE 11

EFFECT OF CHYMOTRYPSINOGEN, ACTIVATED BY CRYSTALLINE TRYPSIN, ON HEMAGGLUTINATION
(incubation for three hours at 37°C.)

Chymotrypsinogen	Trypsin	Sample	EWTI (1 pct.)	HA
1 ml 1%	0.1 ml 1%	2 ml 40-5	0.1 ml	1280
1 ml 1%	0.1 ml 1%	2 ml 42	0.1 ml	160
1 ml 1%	0.1 ml 1%	2 ml NAF	0.1 ml	0
0.25 ml 1%	0.1 ml 1%	2 ml 40-5	0.1 ml	1280
0.25 ml 1%	0.1 ml 1%	2 ml 42	0.1 ml	160
0.25 ml 1%	0.1 ml 1%	2 ml NAF	0.1 ml	0
1 ml 1%	.....	.....	.....	0

It cannot be stated unequivocally that chymotrypsin produces a modification of IBV like that produced by T. Although inactivation was attempted, the possibility of residual chymotrypsin cannot be discounted since the enzyme, in itself, agglutinated red cells.

Turbidimetric determination of hemagglutination. In the standard T process a visible turbidity was produced upon addition of EWTI to the T-IBV mixture. In an attempt to correlate optical density and HA, a Beckman Model B spectrophotometer was used and optical density was measured at a wave length of 500 mμ. The results are presented in Tables 12, 13, and 14, and in Figure I.

Hemagglutination varied directly with optical density and ID_{50}.

No turbidity is evidenced if 2×T and EWTI are mixed. The turbidity appears only upon mixing T and EWTI. Since T and not EWTI is present during the initial incubation of the standard T process, a turbidity curve of T-EWTI was plotted maintaining EWTI constant. The results are presented in Table 15 and in Figure II.

The production and nature of the precipitate in the T-IBV-EWTI complex was considered to be the result of one of the following: (1) a reaction between free T and EWTI; or (2) a reaction between EWTI and some product or products of T digestion.

TABLE 12

OPTICAL DENSITY AND HA TITER OF IBV STRAINS IN STANDARD T PROCESS

Strain	HA Titer	Optical Density
1-7	640	0.24
3-6	1280	0.32
40-5	1280	0.42
40-5	2560	0.48
41-1	2560	0.50
2-11	5120	0.62

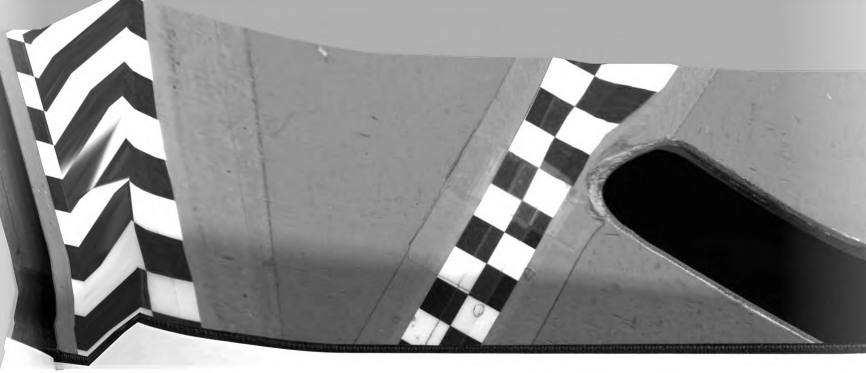


TABLE 13

OPTICAL DENSITY AND HA TITER OF IBV STRAINS IN STANDARD T PROCESS AT DIFFERENT INCUBATION PERIODS

Strain	Time of Incubation	HA Titer	Optical Density
40-5	1 hour	1280	0.26
40-5	2 hours	5120	0.31
40-5	3 hours	10240	0.42
40-5	3 hours	10240	0.46
40-1	3 hours	0	0.055
40-25	3 hours	2560	0.28

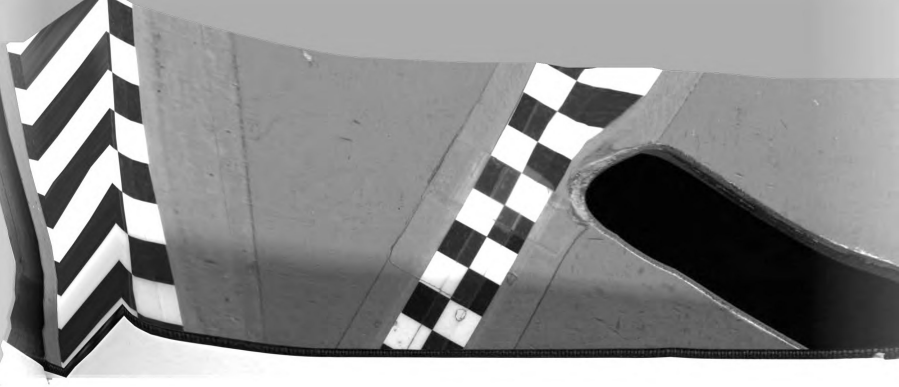


TABLE 14

OPTICAL DENSITY, HA TITER, AND INFECTIVITY OF DILUTIONS OF IBV 40-5 IN STANDARD T PROCESS

Dilution	HA Titer	Optical Density	Embryo Infectivity Prior to Treatment (log 10/0.1 ml)
None	2560	0.40	7.0
1:2	640	0.30	6.7
1:4	20	0.185	6.4
1:8	10	0.10	6.1
1:10	0	0.078	6.0
1:16	0	0.05	5.8

FIGURE I

HEMAGGLUTINATION TITER AND OPTICAL DENSITY
OF IBV 40-5 AFTER STANDARD T PROCESS

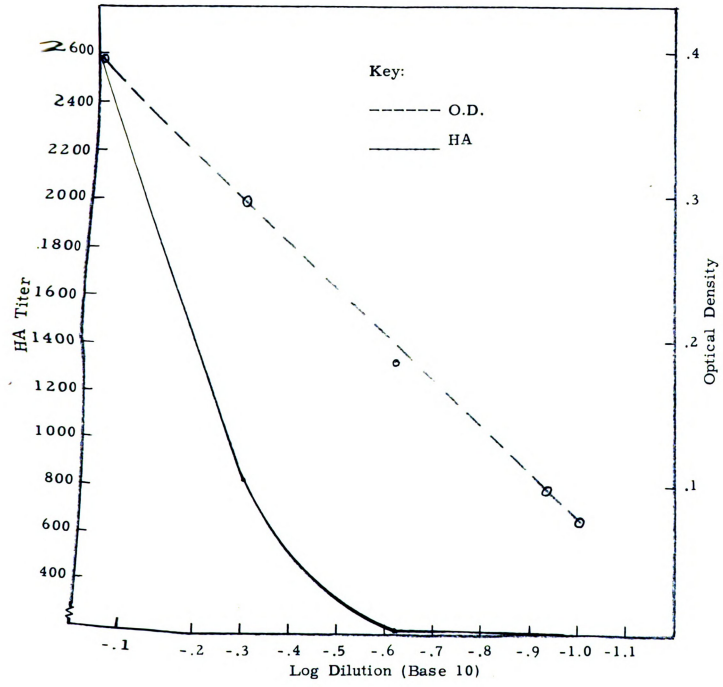


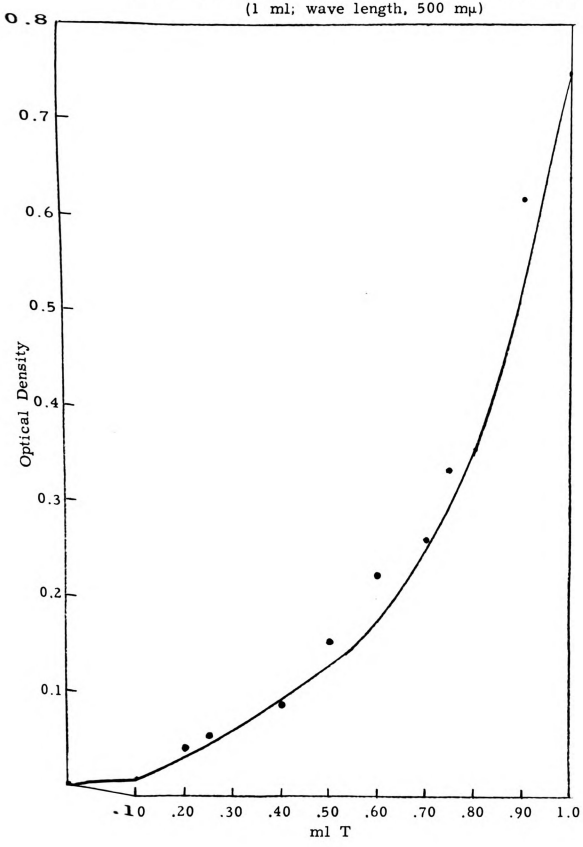
TABLE 15

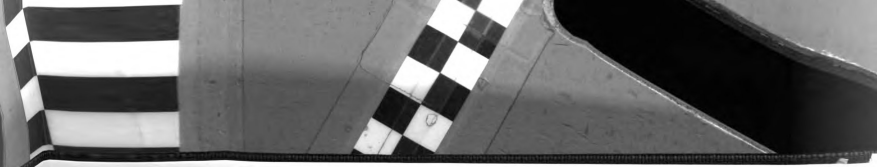
OPTICAL DENSITY OF MIXTURES OF T AND EWTI
(wave length, 500 mμ)

Tube No.	1% T (ml)	Distilled Water (ml)	1% EWTI (ml)	Optical Density
1	1.0	2.0	1.0	0.75
2	0.9	2.1	1.0	0.62
3	0.8	2.2	1.0	0.36
4	0.75	2.25	1.0	0.34
5	0.7	2.3	1.0	0.267
6	0.6	2.4	1.0	0.23
7	0.5	2.5	1.0	0.16
8	0.4	2.6	1.0	0.095
9	0.25	2.75	1.0	0.065
10	0.2	2.8	1.0	0.05
11	0.1	2.9	1.0	0.023
12	. . .	3.0	1.0	0.00
13	1.0	3.0	. . .	0.00

FIGURE II

OPTICAL DENSITY OF MIXTURES OF T AND EWTI
(1 ml; wave length, 500 mμ)



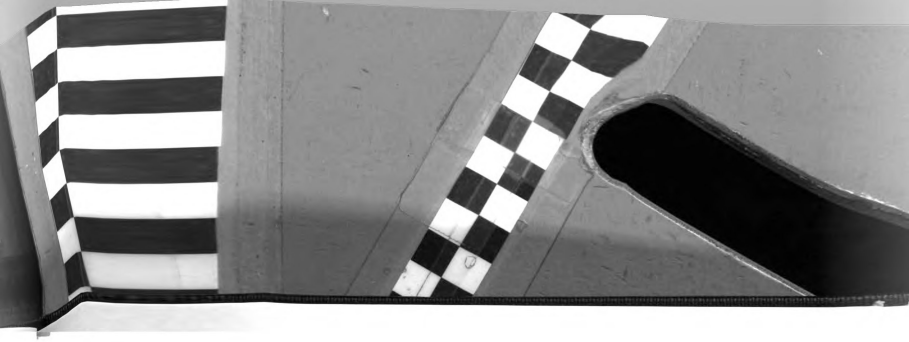


For further information on the nature of the turbidity, T was added to NAF, IBV 40-5, and IBV 42, followed immediately by addition of EWTI. No turbidity was evident immediately in any of these mixtures, but after 15 to 20 minutes turbidity appeared with IBV 40-5. This is in keeping with the rate of appearance of the precipitate in the standard process.

Later trials with a solution that contained 0.25 per cent T prior to filtration, and which produced HA titers comparable to those of 1 per cent T, produced no precipitate.

It would appear that T has an affinity for some substrate or inhibitor present in NAF. It might be concluded that IBV 42, the egg-adapted strain, does not act on allantoic fluid to provide alteration of the substrate. The metabolic processes or particulate nature of IBV 40-5 may produce a change in the substrate or act competitively to bind T.

One explanation of the hemagglutination produced by T-treated NAF or IBV 42 in the absence of EWTI may be the direct adsorption of the T-substrate complex on the red cell surface. A second possible explanation is an actual disruption of the T-substrate complex and the preferential adsorption of T onto the red cell. Thus, the T-red cell combination would provide the more favorable energy



state. The complexity of the red cell surface tends to lend credence to either hypothesis.

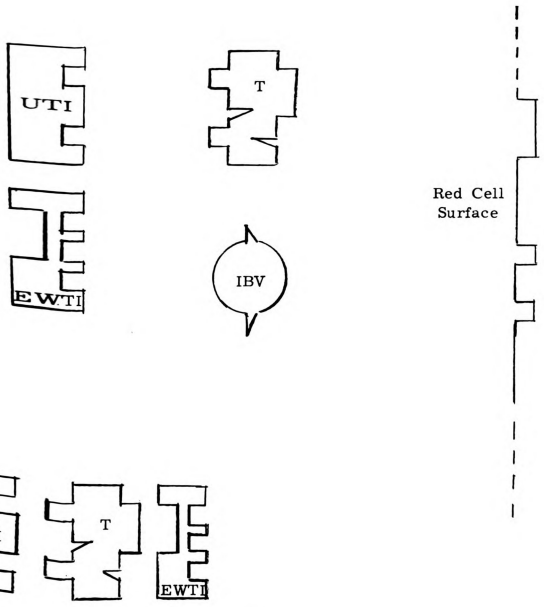
The term "substrate," which is generally used in discussion of enzyme activity, has been used to connote that agent in the allantoic fluid which combines with T. The term "inhibitor" may be more properly applied. Urine is one of the naturally occurring T inhibitors. The allantoic sac serves as a receptacle for kidney excretions of the embryo. The concentration of urea in allantoic fluid increases up to the fourteenth day of incubation. Uric acid content increases throughout the entire incubation period. Creatinine is also present in the allantoic fluid as are inorganic ions. Thus, the components of urine are present in NAF.

Therefore, introduction of T into NAF would result in the combination of T and inhibitor to form an enzyme-inhibitor (EI) complex which does not precipitate. Addition of a second inhibitor, EWTI, provides no stimulus for the dissociation of this initial EI complex which represents a more favorable energy state than that of T-EWTI which precipitates.

Reactive sites as presented in Figure III indicate the possible means of combination of T with urine trypsin inhibitor (UTI), EWTI, virus particles, or red cell surface.

FIGURE III

SCHEMATIC REPRESENTATION OF REACTING PRODUCTS IN THE IBV-HA SYSTEM



T: Trypsin
UTI: Urine Trypsin Inhibitor
EWTI: Eggwhite Trypsin Inhibitor
IBV: Infectious Bronchitis Virus

The T-UTI union is postulated as occurring in only one manner, whereas two modes of reaction are postulated for T-EWTI. One mode of reaction is postulated for the T-IBV union. Consideration must be given to a number of possible reactions between T and red cells, two of which are represented.

The true nature of the components cannot be represented since only two planes are depicted, and, further, the true nature and configuration of the reactive groups, other than the initial sites of reaction of T with substrate, are not known.

The reagents as depicted serve as the basis for the following reactions:

1. The T-UTI union is possible and this combination could be adsorbed to the red cell due to the exposed group on the T molecule.
2. The T-EWTI union occurs and a precipitate is formed which is not adsorbed to the red cell.
3. UTI or EWTI is not adsorbed to the cell and does not cause agglutination.
4. UTI-T-EWTI represents a soluble complex which is not adsorbed on the cell surface.
5. T-IBV represents a true enzyme-substrate complex and the resultant modification of IBV provides a product

which is adsorbed to the cell surface and causes agglutination.

T, therefore, has an affinity for both IBV and UTI present in the infected allantoic fluid. It preferentially reacts with the virus, is released, and reacts with more virus. The addition of EWTI serves to create an excess of inhibitor. The enzyme-substrate cycle is interrupted as enzyme, released from the enzyme-virus complex, is now bound in an EI complex with EWTI as evidenced by formation of the precipitate. The rate of formation of the precipitate indicates the plausibility of this explanation.

The amount of precipitate, as determined spectrophotometrically, varies directly with the hemagglutination titer and would preclude the possibility that hemagglutination was produced by T. It also reflects increased viral concentration as determined by dilution studies.

It may be concluded that hemagglutination exhibited by T-IBV is either the product of adsorption of modified virus or of soluble degradation products derived from the virus through tryptic digestion or a combination of both.

Adsorption Measured by Hemagglutination

Rate of adsorption. Dilutions of IBV were prepared to contain twenty or twenty-five HA units per 0.25 ml. One milliliter each of this preparation, HA buffer, and a 0.5 per cent red cell suspension were placed in tubes and incubated for varying periods at 4°, 23°, and 37°C. All reagents were at thermal equilibrium at the time of combination. At the termination of the contact period the suspensions were centrifuged at 3,000 r.p.m. for ten minutes at 4°C. The cell-free supernatant fluid was removed and tested for hemagglutinative activity. The results appear in Table 16.

Adsorption was complete within five minutes at 23° and 37°C. At 4°C. adsorption was not complete within 25 minutes.

Maximum adsorption. In an attempted determination of maximum adsorption, constant amounts of cells were used with increasing HA units.

A T-IBV preparation with an HA titer of 2560 was diluted 1:5 to contain 512 HA units per 0.25 ml. Multiple volumes of this concentration were used. The results are presented in Table 17.

There is but slight decrease in the ratio of adsorbed to nonadsorbed hemagglutinin with increasing HA units within the range studied.

TABLE 16

RATE OF ADSORPTION OF IBV 40-5 HEMAGGLUTININ (20 and 25 units) BY RED CELLS
(titers determined from supernatant fluid after deposition of cells)

Temperature (°C.)	Hemagglutinin	HA Titer after Adsorption				
		0 Min.	5 Min.	10 Min.	20 Min.	25 Min.
37	Adsorbed with red cells	0	0	0	0	0
	Nonadsorbed control	25	25	25	25	25
23	Adsorbed with red cells	0	0	0	0	0
	Nonadsorbed control	25	25	25	25	25
	Adsorbed with red cells	0	0	0	0	0
	Nonadsorbed control	20	20	20	20	20
4	Adsorbed with red cells	5	5	5	5	5
	Nonadsorbed control	25	25	25	25	25

TABLE 17

TOTAL ADSORPTION OF IBV 40-5 HEMAGGLUTININ BY RED CELLS AT 23°C.

Reaction System			HA Titer of Supernatant Fluid	Total Adsorption (HA units)	Pct. Total Hemagglutinin Adsorbed
HA Units	HA Buffer	0.5 pct. Red Cell Suspension			
512 (0.25 ml)	0.75 ml	0.25 ml	10	512	98
1024 (0.5 ml)	0.5 ml	0.25 ml	20	1004	98
1536 (0.75 ml)	0.25 ml	0.25 ml	40	1496	97
2048 (1.0 ml)	0.00 ml	0.25 ml	80	1968	96

The adsorption is similar to that described by Magill (71) for influenza virus. The forces of attraction between hemagglutinin and chicken erythrocytes are governed by an orderly mechanism which effects a proportional distribution of hemagglutinin between erythrocytes and suspending fluid.

Adsorption Measured by Viral Infectivity

Effect of trypsin on chicken embryos. For anticipated studies on the influence of T on viral infectivity, it was necessary to establish the effect of T and EWTI on embryos.

Five embryos were inoculated via the allantoic cavity (0.1 ml per egg) with serial tenfold dilutions of T, EWTI, T followed by EWTI, and a mixture of equal parts of T and EWTI.

Embryo mortality within 24 hours was produced by 0.3 mg of T. One milligram of EWTI produced no visible effect on the embryos as observed seven days after inoculation. The mixture of T and EWTI was innocuous. However, if 0.3 mg or more of T was inoculated and an equal amount of EWTI inoculated within 30 to 60 seconds later, the T expressed its lethality and embryo mortality occurred.

This lethal effect of T seemingly is not in keeping with the earlier hypothesis that T combines with an inhibitor present

in NAF. This discrepancy may be resolved in any of three ways:

1. There is an increase in urea in NAF up to the fourteenth day. Urate concentration and total nitrogen increase throughout incubation. The embryos were inoculated on the ninth day. It is possible that inhibitor may be present in lesser amounts than that contained in the twelve-day allantoic fluid used in the standard T process. The inhibitor can then inactivate only a portion of the T introduced, thereby allowing the residual T to be adsorbed to cells of the embryo and cause death.

2. The T-UTI complex may be toxic.

3. The enzyme may selectively react with the embryonic cells.

Effect of trypsin on viral infectivity. For application in future adsorption studies, the effect on viral infectivity of the T and EWTI contained in the standard T process was determined. Strain IBV 40-5 with ID_{50} 7.0 retained the same titer after incubation at 37° C. for three hours. The virus, as contained in the T-IBV-EWTI mixture, had an ID_{50} 5.3. These data indicate a decrease of 1.7 log units or a fifty fold reduction of infectivity due to treatment of the virus in the procedure.

Adsorption and infectivity. The adsorption procedure consisted of combining 1 ml of T-IBV-EWTI with 1 ml of a 0.5 per cent red cell suspension. This mixture was incubated at 23°C. for 10 minutes and then centrifuged at 3,000 r.p.m. for 10 minutes at 4°C. The supernatant fluid was removed and its ID_{50} determined.

The EWTI and red cell suspension used were not sterile, so antibiotics (10,000 units of penicillin and 10 mg of streptomycin per milliliter of preparation) were added. All dilutions have been considered in the expression of concentration in the following:

1. Untreated virus ID_{50} 6.2.
2. Virus after incubation at 37°C. for three hours ID_{50} 6.2.
3. Virus after standard T process ID_{50} 4.8.
4. Virus after standard T process and adsorption by red cells ID_{50} 4.3.

The reduction in viral concentration after combination with red cells implied that at least part of the hemagglutinin is intimately bound to the virus particle or that the virus particle itself serves as the hemagglutinin.

Relationship between infectivity titer and hemagglutination titer. If it is assumed that one virus particle represents one infective dose, values may be obtained such that HA units may be expressed

in terms of infectivity units and a relationship between HA units and red cell concentration may be established.

The ID_{50} of untreated virus was 6.2, or 1,581,000 infective doses per 0.1 ml. The HA titer was 2560 per 0.25 ml. By conversion, $(2.5 \times 1{,}581{,}000)/2560 = 1544$ active particles per HA unit.

This does not consider the inactive particles which may have been present and which may be modified by T to serve as hemagglutinative units.

The 0.5 per cent red cell suspension contained approximately 7,500,000 cells per 0.25 ml as determined by direct count using a hemacytometer. If the total number of red cells are considered reactive, then one particle must serve as hemagglutinin for 4,858 cells. It would be physically impossible for one virus particle the size of IBV to serve as the hemagglutinative bond for this number of cells.

If the initial assumption, upon which the calculations are based, is accepted, then it must be concluded that something other than the viral particle enters into the phenomenon. Assuming the exposure of a specific hemagglutinin associated with IBV, the most likely conclusion would be that part of the hemagglutinative activity is intimately bound to the particle, since there is a diminution in infectivity titer after adsorption, whereas the greater portion of the

activity is due to a hemagglutinin distinct from the particle per se. This would parallel the hemagglutinative activity of vaccinia virus.

Further Characterization of the Infectious Bronchitis Virus Hemagglutinin

Elution. The progression of hemagglutination and subsequent elution of IBV in the HA test was studied at 4°, 23°, and 37°C. As shown in Table 18, there is a direct correlation between the rate of adsorption and an indirect relation between the rate of elution with respect to temperature.

The adsorption of IBV appears to be that of chemisorption.

Hemagglutination by different strains of infectious bronchitis virus. IBV 40-5 was used almost exclusively for the development of the standard T process but it was also ascertained that other strains could participate in the reaction. The hemagglutinative activity of certain other strains is presented in Table 19. All strains except IBV 42, the egg-adapted strain, were modified and yielded high HA titers.

Hemagglutinative activity and initial recovery of infectious bronchitis virus in chicken embryos. When isolation of IBV from infected chickens is first attempted in chicken embryos, it is

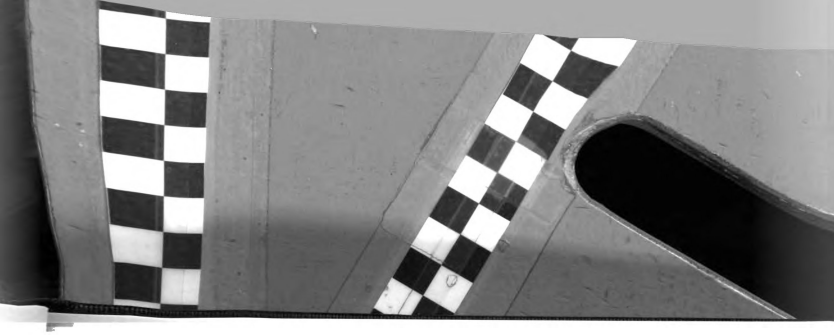


TABLE 18

PROGRESSION OF ADSORPTION AND ELUTION IN HEMAGGLUTINATION BY IBV 40-5

Time	4°C.	23°C.	37°C.
30 min.	Incomplete and indistinct	2560	2560 Most rapid settling of cells and clearest patterns
45 min.	Incomplete and indistinct	2560	2560
60 min.	Still indistinct but read as 1280	2560	1280
80 min.	640	2560	1280
14 hrs.	Elution in dilutions greater than 1:20	Elution in dilutions greater than 1:320	No elution Some hemolysis

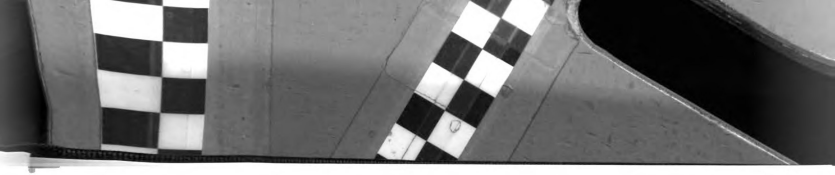


TABLE 19

HEMAGGLUTINATION BY STRAINS OF IBV AFTER MODIFICATION IN THE STANDARD T PROCESS

IBV Strain	HA
40-5	2560
42	0
1-7	2560
2-10	5120
3-6	2560
16-34	1280
19-5	1280
20-10	2560
21-8	640
23-15	640
24-10	2560
41-1	5120

sometimes necessary to make more than one passage and rarely more than three passages before typical gross lesions of the embryo are manifest.

To correlate hemagglutinative activity of T-IBV with egg passage and positive reaction of the embryo, three different strains were used. The results in Table 20 show that with strain 40 and 40-5-1C three egg passages were necessary before HA and viral activity were demonstrated. Strain 41 was positive for both reactions on the first passage. These data indicate that hemagglutinative activity is associated with viral concentration.

Effect of trypsin on red cells. To 2 ml of a 25.0 per cent saline suspension of red cells, 1 ml of 1 per cent T was added and the mixture was incubated at 37°C. for three hours. One milliliter of EWTI was added and the cells were washed in saline through three cycles of differential centrifugation. The cells were then used in the HA test with both modified and untreated IBV 40-5 and IBV 42 and NAF.

The data in Table 21 show that IBV does not agglutinate trypsin-treated red cells in contrast to agglutination of untreated cells as used in the HA test. This indicates that HA as observed in the test is due to trypsin-modified IBV and not to trypsin-modified cells.

TABLE 20

CONCENTRATION OF IBV HEMAGGLUTININ IN EARLY EGG PASSAGE

Strain and Passage	Mortality or Gross Lesions	HA Titer
40-1	2/10	0
40-2	2/10	0
40-3	7/10	5120
40-5-1C-1[a]	1/10	0
40-5-1C-2[a]	2/10	0
40-5-1C-3[a]	8/10	2560
41-1	7/10	5120

[a] IBV 40-5 was used to infect three-week-old chickens. IBV was recovered from lung and tracheal suspensions and inoculated into eggs. The last number indicates egg passage.

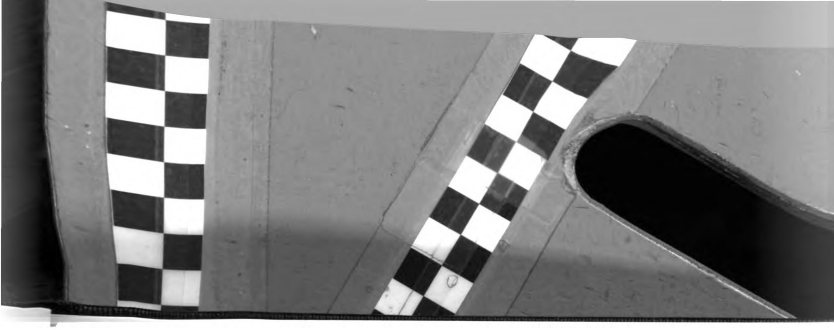


TABLE 21

TRYPSIN-MODIFIED RED CELLS IN HEMAGGLUTINATION TESTS

Virus	HA Titer	
	Untreated Cells	T-modified Cells
T-IBV 40-5	5120	0
T-IBV 42	0	0
T-NAF	0	0
Untreated IBV 40-5	0	0

Thermostability of the hemagglutinative activity of infectious bronchitis virus. Infected and normal allantoic fluids were heated for certain periods at 56°C. prior to use in the standard T process. The results are presented in Table 22.

As measured by infectivity titration, IBV 40-5 resists 56°C. for 120 minutes (27). These data indicate that heat-inactivated virus remains susceptible to tryptic modification. Continued heating extends denaturation and the resultant product does not evidence modification to the same degree as unheated IBV after T treatment.

Virus, after T treatment, was stored at -30°C. for different periods after which its hemagglutinative activity was determined. The results are presented in Table 23.

The results obtained showed such wide variation that no valid explanation can be offered. T-IBV could be stored at -30°C., but without any certainty of stability of hemagglutinative activity.

Random sampling of T-IBV stored at 4°C. for similar periods of time showed essentially the same results.

Colloids. Precipitation reactions of arsenic trisulfide, chromium hydroxide, ferric hydroxide, red colloidal gold, and silver colloids when combined with the reagents listed in Table 24 were of no value as indicators of charge of IBV.

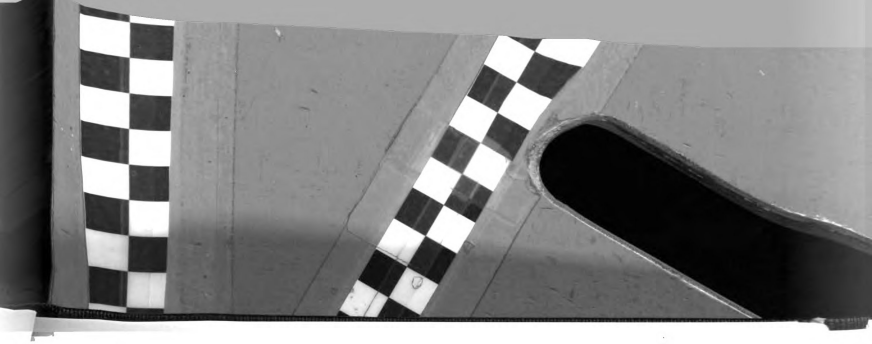


TABLE 22

THERMOSTABILITY OF IBV-INFECTED AND NORMAL ALLANTOIC FLUIDS PRIOR TO STANDARD T PROCESS

Time (min.) at 56°C.	HA Titer		
	40-5	42	NAF
0	640	0	0
15	640	0	0
30	640	0	0
60	640	0	0
90	640	0	0
120	640	0	0
150	640	0	0
180	160	0	0

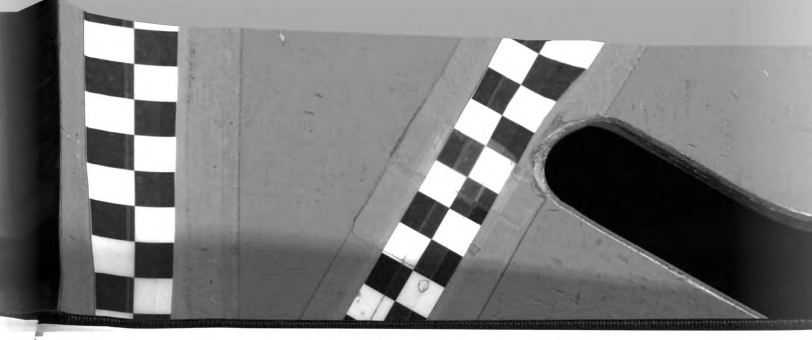


TABLE 23

THERMOSTABILITY AT -30°C. OF THE HEMAGGLUTINATIVE ACTIVITY OF IBV AFTER STANDARD T PROCESS

Strain	Initial Titer	Time (days) at -30°C.	Final Titer
40-5	640	1	10
40-5	....	1	10
40-5	640	1	5
40-6	2560	1	40
40-5	2560	2	20
2-11	640	2	640
41-1	1280	2	640
40-3	....	3	5120
2-10	....	3	5120
41-1	....	3	640
40-5	640	4	80
40-5	....	4	640
40 5	5120	8	160
40-5	1280	8	320

TABLE 24

COLLOIDAL PRUSSIAN BLUE AS AN INDICATOR OF THE CHARGE ON REAGENTS IN THE STANDARD T PROCESS

Reagent (0.2 ml added to 2.0 ml of Prussian blue)	Reaction of Colloid
T	Precipitates in 15 minutes
EWTI	Stable at 1 hour
T, EWTI	Precipitates in 1 hour
Distilled water	Precipitates in 45 minutes
NAF	Precipitates in 30 minutes
IBV 40-5	Precipitates in 30 minutes
IBV 42	Precipitates in 30 minutes
T, NAF, EWTI	Stable at 14 hours
T, IBV 40-5, EWTI	Precipitates in 15 minutes
T, IBV 42, EWTI	Precipitates in 15 minutes
None	Precipitates in 1 hour

Colloidal Prussian blue that had been stored for nine months and which exhibited some spontaneous precipitation served to indicate charge differences. Combinations of colloid and reagents were prepared as follows: 0.2 ml of reagent was added to 2.0 ml of the colloid and observed for precipitation. The results appear in Table 24.

The stabilization of the colloid by EWTI and T-NAF-EWTI indicates that each carries a net negative charge for the colloid carries a negative charge also. This activity tends to support the earlier hypothesis that T is bound to an inhibitor upon introduction into NAF in vitro. If the T were free it would combine with the added EWTI and precipitate the colloid as did T-EWTI.

Hemagglutination by peptones, polypeptides, and amino acids. Hemagglutination by the T-modified IBV was the stimulus for investigation of HA by the products of T hydrolysis of other proteins. Serial twofold dilutions of 1 per cent aqueous solutions of trypticase, tryptone, and tryptose were tested for their hemagglutinative activity. The results are presented in Table 25.

Hemagglutination by trypticase and tryptone, but not by tryptose, serves to emphasize the range of hemagglutinative agents and the question of the specificity of the T-induced IBV hemagglutinin.

TABLE 25

HEMAGGLUTINATION BY PEPTONES
(titer expressed as reciprocal of g/ml)

Peptone	Titer	1 Per Cent EWTI Added	Titer
Trypticase	400	0.1 ml/ml	100
Tryptone	800	0.1 ml/ml	800
Tryptose	0	0.1 ml/ml	0

Solutions of L-arginine, glycine, L-lysine, L-methionine, L-phenylalanine, L-serine, and L-tyrosine, alone or in combination, exhibited no hemagglutinative activity.

An aqueous solution of trypticase, glycine, L-arginine, L-lysine, L-metionine, and L-serine, each at 1 per cent final concentration, had an HA titer of 1067 expressed as the reciprocal of grams per milliliter. Since the amino acids in themselves cannot cause agglutination, the titer, based on the weight of trypticase, would be 6400.

Arginine and lysine represent the points of initial tryptic activity, so a solution containing one gram each of trypticase, L-arginine, and L-lysine in 100 ml of water was prepared. The titer

of this mixture was 133, but if based on the weight of trypticase alone it was 400.

From the titers based on the weight of trypticase it is seen that the addition of the five amino acids enhances the hemagglutinative activity, whereas addition of arginine and lysine has no such effect.

The approximate concentrations of metallic ions, amino acids, and vitamins present in the peptone have been determined but the forms in which they exist is unknown so the mode of the reaction of the added amino acids cannot be explained at this time.

Agglutination of red cells of certain species. The hemagglutinative activity associated with most viruses is generally active on the red cells of at least a few species. The red cell spectrum of the IBV hemagglutinin is presented in Table 26.

Hemagglutination by IBV is effective only with chicken erythrocytes.

A 1 per cent solution of 2×T agglutinated human O cells.

A trypticase-amino acid (arginine, glycine, lysine, methionine, phenylalanine, serine, and tyrosine) mixture had an HA titer of 1:100 (g/ml) with both chicken and human O cells.

TABLE 26

AGGLUTINATION OF RED CELLS OF CERTAIN SPECIES BY T-MODIFIED IBV

Species	HA Titer
Chicken	2560
Rabbit	0
Cat	0
Cog	0
Cow	0
Horse	0
Sheep	0
Human O	0

Hemagglutination-inhibition (HI)

Hemagglutination-inhibition by serum. Inhibition of the IBV hemagglutinin was tested with known positive and negative sera. The results appear in Table 27.

The inhibitory effect of the negative serum may be that of a nonspecific nature or it may indicate nonspecificity of the IBV hemagglutinin.

TABLE 27

HEMAGGLUTINATION-INHIBITION OF IBV BY SERA

IBV Strain	HA Titer	HA Titer			
		Alpha Procedure		Beta Procedure	
		Positive Serum	Negative Serum	Positive Serum	Negative Serum
40-5	2560	12800	12800	12800	12800
40-15	1280	6400	6400	12800	12800
40-16	10240	51200	51200	12800	12800
42	320	1600	1600	12800	12800

Inactivation of nonspecific inhibitors in normal chicken serum was attempted by the following treatments which yielded negative results:

1. Inactivation at 56°C. for 30 minutes.
2. Inactivation at 62°C. for 20 minutes.
3. Addition of an equal volume of 3.81 per cent sodium citrate to the undiluted serum used in the beta test.
4. Filtration through Seitz EK pad.
5. Combination of equal volumes of serum and supernatant fluid from Clostridium welchii cultures and incubation at

room temperature for one hour.

6. Ether extraction of the serum at room temperature.

Each of the three types of Clostridium welchii added to positive IB serum produced a precipitate. No precipitate was formed when they were added to negative serum. These sera had been stored at -30°C. from two to six months. The pH of the sera was 8.0, the Clostridium welchii supernatant fluid was 6.0, and the combination was 6.7. A second trial with different cultures of the same strains did not produce this effect. In this latter attempt the pH of the combination was 7.1. Differences in quantity and quality of precipitates of these positive and negative sera were observed upon addition of concentrated hydrochloric acid. Serum containing antibodies against NDV but not IBV reacted in the same manner as did serum containing antibodies against IBV only. The precipitate is apparently an effect of pH and not a specific reaction between components in the system.

Sera were inactivated at 62°C. for 20 minutes and then treated with T and EWTI as in the standard T process. The results appear in Table 28.

Identical results were obtained with positive and negative sera in all tests except with IBV 40-23 and one test with IBV 40-5.

TABLE 28

HEMAGGLUTINATION-INHIBITION OF IBV BY SERA INACTIVATED AT 62°C. FOR 20 MINUTES AND THEN TREATED WITH T AND EWTI

IBV Strain	HA Titer	HA Titer			
		Alpha Procedure		Beta Procedure	
		Positive Serum	Negative Serum	Positive Serum	Negative Serum
40-5	2560	12800	12800	6400	6400
40-5	2560	12800	12800	6400	6400
40-5	2560	.....	.....	1600	1600
40-23	640	3200	3200	800	0
40-5	320	1600	1600	800	50
40-5	2560	.....	.....	6400	6400

The HA titer of IBV used in these two tests was considerably lower than in the other tests which were performed at different times. It is possible that the reduced HA titers of the IBV indicate an incomplete modification which may have resulted from a temperature fluctuation or reduced activity of the trypsin employed. The IBV and sera were processed at the same time so any deviation from the standard would be reflected in the activity of both.

Crystalline trypsin was used later in treatment of sera, and either EWTI or SBTI was used as inhibitor. Positive and negative sera exhibited equal HI titers.

As controls, the sera employed were tested for hemagglutinative activity. Positive and negative sera treated in the following ways showed no hemagglutinative ability:

1. Untreated.
2. Inactivated at 56°C. for 30 minutes.
3. Inactivated at 62°C. for 20 minutes.
4. Standard T process with or without prior heat inactivation.

Spontaneous agglutination by sera treated with 2×T was observed after incubation at 56°C. for 30 minutes even after the addition of an equal amount of EWTI.

The HI test was modified in that the IBV hemagglutinin and cells were combined prior to addition of serum. Positive and negative sera proved capable of disrupting the IBV hemagglutinin-red cell union as evidenced by lack of hemagglutination.

The positive and negative sera were employed in HI tests with the previously used trypticase-amino acid mixture which had been stored at -30°C. for three weeks. The results appear in Table 29.

TABLE 29

HEMAGGLUTINATION-INHIBITION TESTS USING A TRYPTICASE-AMINO ACID MIXTURE

Titer[a]	HI Titer of Sera (alpha procedure)			
	Untreated Sera		Sera Heated at 62°C. for 20 Minutes	
	Positive Serum	Negative Serum	Positive Serum	Negative Serum
1067	0	0	0	0
267	40	40	40	40
67	0	0	0	0
267	20	20	20	20

[a]No virus present.

There is a striking difference between the inhibition of the IBV and the polypeptide hemagglutinin by serum. Hemagglutination by IBV is completely inhibited by sera employed in the alpha procedure, whereas the highest HI titer obtained with the trypticase-amino acid mixture was 40. This indicates a difference in the nature of these hemagglutinins.

Effect of other inhibiting agents. To detect the effect of other agents that might inhibit the IBV hemagglutinin, untreated NAF, IBV 40-20 and IBV 42, and EWTI and SBTI were diluted 1:5 (1:4 in the case of SBTI) and used in place of serum in the alpha procedure of HI tests. NDV was used as a positive control to show agglutination. The results are presented in Table 30.

Normal and IBV-infected allantoic fluids contain inhibitor active against HA by NDV and IBV. Inhibitor is present in greater concentration in the IBV-infected allantoic fluid as measured by NDV HA.

Inhibition of the IBV hemagglutinin by EWTI but not by SBTI indicates further that the hemagglutination is not a result of the activity of residual trypsin. EWTI, which is ovomucoid, represents one of the Francis-type inhibitors which are mucoid in nature. Ovomucoid is not active against the NDV hemagglutinin.

The supernatant fluid from C. welchii, type A, was diluted 1:5 and tested for inhibition of hemagglutination with the IBV hemagglutinin. This preparation caused hemolysis but agglutination was apparent through the 1:80 dilution of virus. It may be concluded that the IBV hemagglutinin is dissimilar to that of vaccinia and ectromelia which is lipoidal in nature and is inhibited by lecithinase (89).

TABLE 30

HEMAGGLUTINATION-INHIBITION
(ALPHA PROCEDURE)

Hemagglutination	HA Titer	Inhibitor	HA Titer after Combination with Inhibitor
NDV	1280		
T-IBV	2560		
NDV	1280	NAF	160
T-IBV	2560	NAF	0
NDV	1280	IBV 42	80
T-IBV	2560	IBV 42	0
NDV	1280	IBV 40-20	80
T-IBV	2560	IBV 40-20	0
NDV	1280	EWTI	1280
T-IBV	2560	EWTI	0
NDV	1280	SBTI	1280
T-IBV	2560	SBTI	2560

DISCUSSION

The action of trypsin on feline pneumonitis virus and the GD VII strain of murine encephalitis results in increased infectivity, presumably by disaggregation of the virus. Trypsin has been employed for purification of psittacosis and other viruses. Trypsin acts to render particulate matter, other than virus, soluble, and virus may be concentrated by centrifugation.

Tryptic digestion reduces infectivity of potato virus X. A tenfold reduction of infectivity of the LEE strain of influenza B was reported following treatment with trypsin. This is the only report of reduction of infectivity of an animal virus by trypsin.

IBV is an animal virus whose molecular structure can be determined in part by the action of trypsin as reflected in reduced infectivity and induced hemagglutinative activity. Trypsin cleaves bonds formed by the carboxyl groups of the basic amino acids, arginine, and lysine. Thus, either arginine and/or lysine is present in the protein moiety of the virus. The spatial configuration of the protein molecule and its position in the virus particle permit formation of the enzyme-substrate complex.

Specificity of the induced hemagglutinin has not been demonstrated by HI tests with anti-IB serum. The mode of inactivation of nonspecific inhibitor present in normal serum has not as yet been ascertained. Normal chicken serum may be rendered panagglutinative through modification by trypsin. Although trypsin has been reported to inactivate nonspecific inhibitor of influenza virus in normal human, rabbit, ferret, mouse, guinea pig, and chicken sera, it has not proved of value as employed in the present studies. In one trial with IBV a difference in modification of normal and immune sera was observed after T treatment. Further investigation of the concentration of T and time and temperature of the reaction may provide for proper modification. The RDE of Vibrio cholerae, which was not available at the time that these studies were conducted, should be utilized as well as sodium and potassium periodate.

The hemagglutinative activity of IBV induced by trypsin is distinguished from that of the peptones tested. Differential inhibitory action of sera is so marked as to indicate a difference in the nature of these two agglutinins.

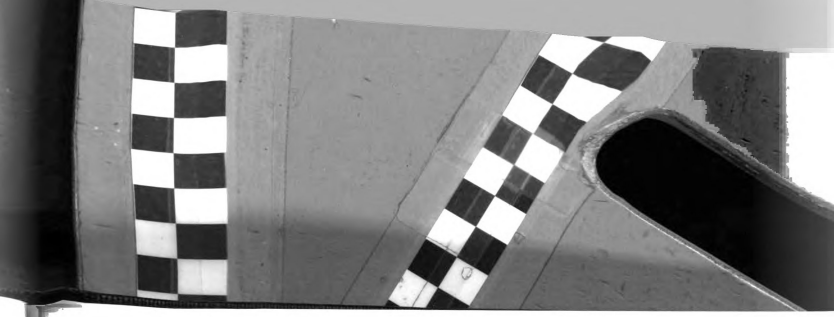
A further distinction in the nature of these two hemagglutinins can be derived from a comparison of the hemagglutination titers on a weight-per-volume basis. The end points of the polypeptide mixtures were determined on the basis of grams per

milliliter and the titer expressed as the reciprocal. A similar evaluation can be made for the virus.

The virus is a sphere of 70 mμ average diameter with a volume of 143,800 $m\mu^3$. One milliliter equals 10^{21} $m\mu^3$. Therefore, if the infectivity and hemagglutination titers determined for a typical IBV strain are employed, the HA titer may be expressed in terms of mass per volume.

The most common IBV HA titer was 2560/0.25 ml and the corresponding infectivity titer was $10^7/0.1$ ml. If it is assumed that one virus particle represents one infectious unit, the total volume of virus may be determined by multiplying the volume of one particle by the total number of particles. Therefore, 143,800 $m\mu^3$ or $10^{5.1577}$, multiplied by 10^8 particles/ml equals $10^{13.1577}$ $m\mu^3$. The volume of the particles in $m\mu^3$ divided by the $m\mu^3$ in one milliliter provides the volume of the virus in milliliters. Therefore: $10^{13.1577}/10^{21} = 10^{-7.8423}$ or 1.438×10^{-8} ml.

If the density of IBV is assumed to be about 1.2, which is within the range reported for other animal viruses of similar size and shape, the weight of the virus in one milliliter of infected fluid is 1.72×10^{-8}. In the HA test 0.25 ml of the IBV dilutions was used. Therefore, 0.25 (1.72×10^{-8}) equals 4.3×10^{-9}. Expressed as the reciprocal of grams per milliliter, the HA titer of IBV is



6×10^{11}, whereas the highest HA titer observed with the peptones was 1067.

It was previously hypothesized that one infectious unit represented 1,000 virus particles. Agglutination then could be more plausibly attributed to the IBV particles. If this number of virus particles is considered, the HA titer of IBV would only be reduced to 6×10^{8} and there is still great distinction between the induced IBV hemagglutinin and that of the peptones.

The true physical nature of the induced IBV hemagglutinative agent and its reaction with red cells possibly may be determined by differential ultrafiltration and electron microscopy.

No enzymic quality of the virus itself is involved in the hemagglutinative activity. This is evidenced by heating IBV. The HA titer remained constant after 150 minutes at 56°C. and a reduction in titer appeared only after 180 minutes. The virus is rendered noninfectious after 120 minutes. It may be concluded that denaturation of the virus protein proceeds at a rate such that after 180 minutes there is modification which enhances or limits the activity of trypsin either by alteration of initial specific substrate or secondary sites of reaction.

The carbohydrate portion of ovomucoid represents 20 per cent of its substance and consists of three molecules of mannose,

seven of acetylglucosamine, and one of galactose (71). The ovomucoid may serve as a reducing substance through the action of the sugars.

That reduction alone is not the criterion for inhibition of hemagglutination was demonstrated when a preparation of EWTI, which had been stored for two months and had lost some of its activity, was used in the alpha HI procedure. The initial HA titer was 2560. With the ovomucoid the titer was 320, and not the expected zero. When a preparation representing a 1:5 dilution of 1 per cent each of EWTI and sodium thioglycollate was used as inhibitor the titer was 40. Sodium thioglycollate alone was not inhibitory. The action of sodium thioglycollate was directed to the hemoglobin. The cells were characteristically agglutinated at a virus dilution of 2560 but had a green color which was probably due to the formation of sulfhemoglobin.

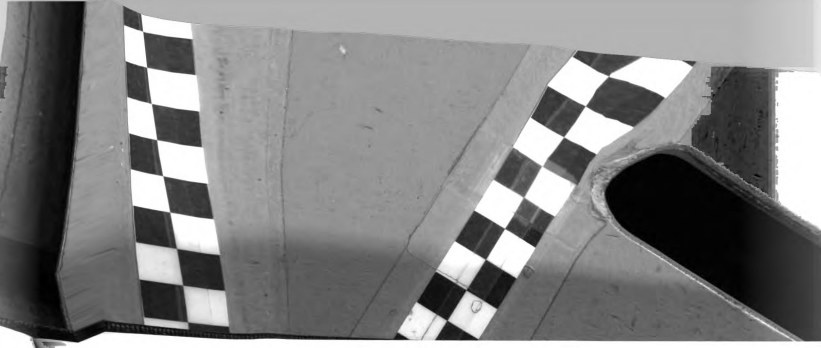
A further consideration of the T-EWTI union and the inhibition of HA by EWTI in this test system may be predicated on the direct variance of turbidity and HA titer. It was postulated that the trypsin which is free to combine with EWTI is that which was reactive with the virus since no turbidity is evidenced in the control fluid. The greater the trypsin reactive with virus, the greater the precipitation of the T-EWTI complex. The reduction in concentration of

EWTI represents a reduction in inhibitor of HA with a resulting higher titer.

The IBV hemagglutinin differs from that of influenza virus as seen in the rates of adsorption at different temperatures. Influenza virus is rapidly and most completely adsorbed to red cells at 4°C., whereas the maximum rate and total adsorption of IBV is greater at 37°C. than at 4°C. Adsorption of influenza virus is characteristic of physical adsorption, whereas that of IBV is more representative of chemisorption.

The HA reaction of IBV differs further from that of the MNI group in that spontaneous elution does not occur.

It is generally considered that the prevailing forces involved in HA are electrostatic in nature. Ion-dipole or the lesser magnitude Van der Waals' forces could account for this adsorption and the tenuous nature of such bonds, particularly amidst the complexity of configuration and radicals in the protein moiety, could be reflected in elution. That elution occurs at 37°C. and not at 4°C. supports the existence of dipole forces in that thermal agitation would serve to disrupt these bonds. This type bond would also account for the more rapid and complete adsorption at lower temperatures.



This mode of reaction does not, in itself, provide for the observed reduction in net negative charge on the surface of the erythrocyte after adsorption and elution of the virus.

Elution of influenza virus is considered to be the result of enzymatic destruction of cellular receptors by the virus. The net activity on the red cell is one of reduction as evidenced by the altered electrophoretic velocity and agglutinability. That the cells are not reagglutinable by the same strain of virus or those lower on the receptor gradient scale may indicate that the enzymatic mechanism is that of hydrolysis rather than of oxidoreduction which would be reversible in this system.

It may be deduced from the over-all reaction of influenza virus HA that positively charged units, whether they be ionic or polar, are necessary for adsorption. Spatial configuration is a determining factor since interionic attractive forces vary as r^{-2}, whereas interdipole attraction varies as r^{-6} or r^{-7}.

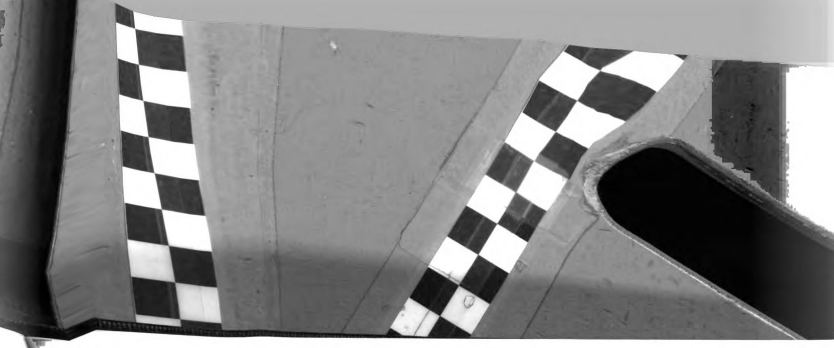
The activity of cations in the adsorption phenomenon may be a combination reaction with viral or blood cell protein. Proteins combine preferentially with calcium, magnesium, phosphate, and bicarbonate ions. The stability of the bond formed between protein molecules and calcium or phosphate ions is due to the high electrostatic action of the bivalent inorganic ions. Univalent ions

such as those of sodium, potassium, or chlorine are less firmly bound. In the process of adsorbing counterions the ionized groups of the protein become neutralized and the over-all effect is a modification of the electrochemical properties of the protein.

If calcium combined with red cell protein there would be a reduction in net negative charge which would result in diminished forces of repulsion between the virus particle and the red cell since both virus and cell carry a net negative charge.

Untreated IBV is incapable of agglutinating red cells. The T-modified IBV has hemagglutinative activity. The initial sites of tryptic activity are peptide bonds involving the carboxyl groups of arginine or lysine. Further enzymatic digestion proceeds but the sites of reaction are not known.

From the initial reaction it is seen that the epsilon amino group of lysine and/or the guanido group of arginine may become exposed. Substitution reactions with proteins indicate that at least a portion of the hydroxyl groups of hydroxyamino acids, the imidazole rings of histidine, and the guanido groups of arginine exist in the form of "free" reactive groups. The HA reaction is conducted at pH 7.2, and the epsilon amino group of lysine would carry a positive charge despite an assumed decrease in the pK_3 of lysine



based on observation of the pK_2 of glycine as a free amino acid and the peptide, hexaglycine.

The positively charged groups could then form salt bridges with surface carbonyl groups of cellular protein. Relatively small numbers of these positively charged groups, as compared with dipoles, would have to be exposed to provide the stability which is manifest.

Elution of the IBV hemagglutinin is probably not a true elution but is due to the tenuous nature of the lattice formation. This elution, as reflected by compaction of cells, occurs only in the higher dilutions of IBV and stems from the nature of the proportional distribution of the hemagglutinin among the erythrocytes. This is not the true compaction of nonagglutinated cells and does not exhibit ready streaming of cells when the tubes are tilted. Streaming is readily evident in untreated cells and those from which NDV has eluted. This further tends to demonstrate a difference in the nature of the bonding of the IBV hemagglutinative agent to the red cell.

It has not been conclusively demonstrated that the egg-adapted strain will react to the same extent in this system as other strains of IBV. In three tests in which the egg-adapted strain had an infectivity titer of $10^7/0.1$ ml only one had an HA titer of 320 after treatment, whereas the other two had no hemagglutinative activity.

Consideration of one aberrant effect of the egg-adapted strain as expressed in high dilution upon embryo inoculation presents the possibility of the involvement of a factor other than that of concentration. This effect is the curling and dwarfing of the embryo rather than the lethal effect which is characteristic of egg-adapted strains. Foregoing any attempted explanation of its existence, cognizance must be given to the possible presence of an agent other than egg-adapted IBV or the presence of a modified form. Back-mutations of the strain have not been reported. If this agent is truly present, it may be responsible for the low HA titers sometimes exhibited by the egg-adapted strain. The reduced or absent hemagglutinative activity may be another expression of adaptation.

Loss of pathogenicity and antigenicity for chickens is characteristic of egg-adaptation of IBV and may likely be the result of structural changes in the virus protein. The lack of response to tryptic activity by the egg-adapted strain may be a further reflection of altered protein structure. The protein molecule of IBV may be elongate with exposed sites that are reactive with T and exposed groups that are essential for pathogenicity and antigenicity. The protein structure of the egg-adapted strain may be altered as a result of folding over of the polypeptide chains resulting in a laminate, globular molecule. The spatial configuration of this laminated

molecule may then result in the masking of these reactive sites and thereby account for altered activity.

Another possible explanation for the differential hemagglutinative activity of IBV and the egg-adapted strain may be based on the differential pathological effects of the two strains. The egg-adapted strain is more virulent and is lethal to embryos within 48 hours. From this greater virulence it may be deduced that a lesser number of particles represent an infective unit of egg-adapted virus than less-well-adapted strains of IBV. Therefore, identical infectivity titers of the two strains would not necessarily represent equality of total virus protein. If this is the case, the lesser hemagglutinative activity of the egg adapted strain would merely be a reflection of lesser substrate.

Total nitrogen determinations (Kjeldahl) of NAF, IBV 40-5, and IBV 42 (egg-adapted) for distinction and correlation of virus protein and hemagglutination were nonconclusive.

The hemagglutinative activity of IBV is dependent on viral concentration and may possibly be utilized as a diagnostic aid for the detection of the virus in embryos inoculated with tissue suspensions. The test is not presently adapted for assay of IBV antibodies. The induced hemagglutinative activity and the concurrent reduction of infectivity are unique to this virus at this time.

So far as known, this is the first evidence presented on the participation of IBV in a hemagglutination system and possible explanation of chemical configuration of IBV protein.

SUMMARY

1. Infectious bronchitis virus was modified by trypsin so that it agglutinated chicken erythrocytes. Trypsin at a final concentration of 0.33 per cent was added to the virus-infected allantoic fluid and incubated for three hours at 37°C. Eggwhite trypsin inhibitor was added so that the final concentrations of trypsin and inhibitor were equal. This preparation contained the active hemagglutinating agent.

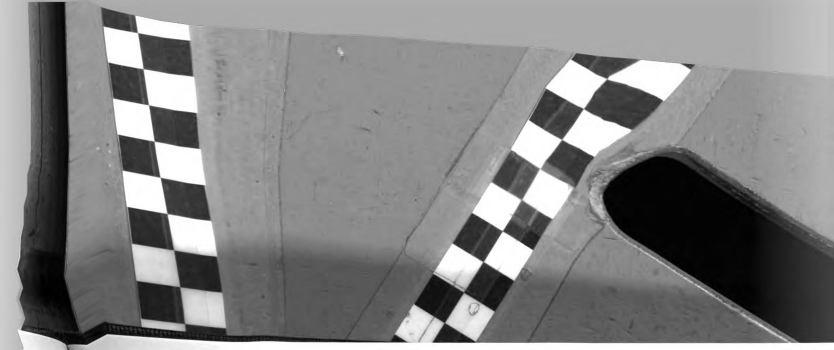
2. A 98 per cent reduction in infectivity for embryos accompanies trypsin modification.

3. The egg-adapted strain does not react to as great an extent in this system as do less-well-adapted strains.

4. The rate and degree of adsorption of the hemagglutinin varies directly with temperature. Elution varies indirectly with temperature.

5. The hemagglutinin is present in two fractions: one in close association with the virus particle, and the second, which constitutes the greater part, is distinct from the particle.

6. Hemagglutination varies directly with viral concentration.



7. There is a direct correlation between the optical density and the hemagglutination titer of the modified virus preparation.

8. Hemagglutination by infectious bronchitis virus is readily inhibited by positive and negative sera, ovomucoid, and normal and infectious bronchitis virus-infected allantoic fluid.

9. Some tryptic digests of casein have hemagglutinative activity.

10. The infectious bronchitis virus hemagglutinin is distinct from that of other viruses and peptones and lipids which have been demonstrated to have hemagglutinative activity.

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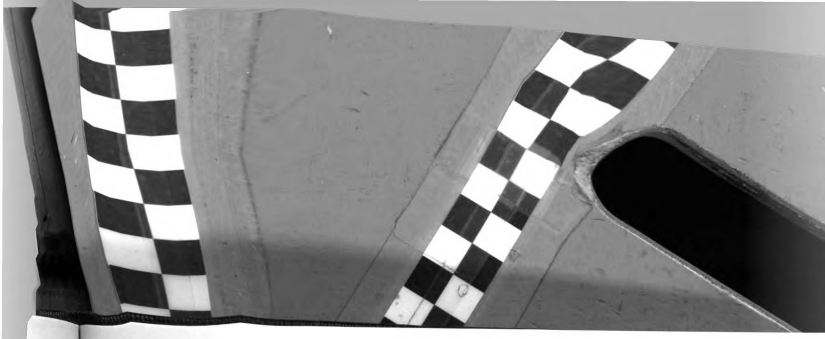

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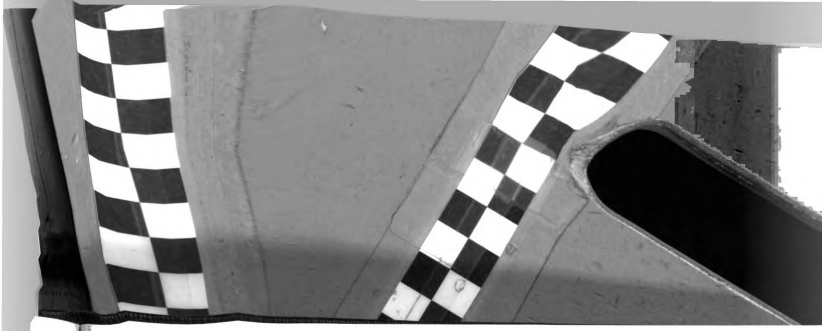

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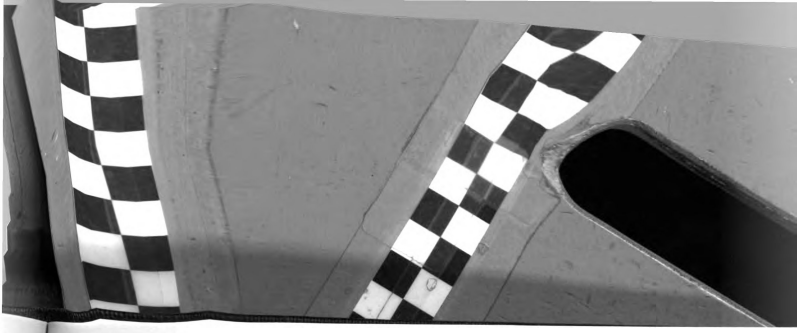

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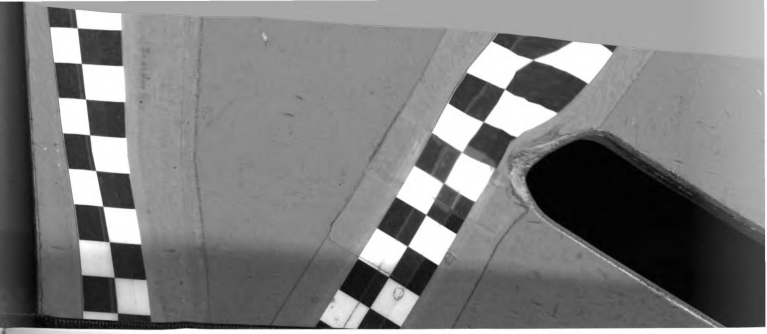

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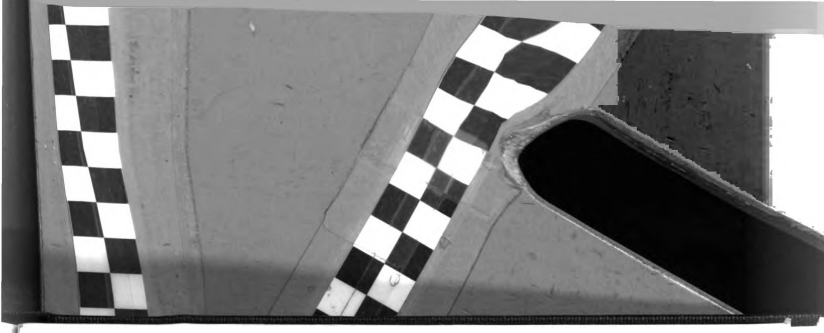

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