STUDIES OF THE DISSOCIATION OF INFECTIOUS BRONCHITIS VIRUS-ANTIBODY COMPLEX

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
Mark F. Stinski
1966

STUDIES OF THE DISSOCIATION OF INFECTIOUS BRONCHITIS VIRUS-ANTIBODY COMPLEX

By

Mark F. Stinski

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Microbiology and Public Health

1966

ACKNOWLEDGEMENTS

My sincere thanks to Dr. Charles H. Cunningham, Professor of Microbiology and Public Health, for his guidance throughout the investigation and the preparation of this manuscript.

I wish to express my appreciation to Mrs. Martha Spring and Mr. Robert B. Lacey, Department of Microbiology and Public Health, for their inspiration and advice.

I am greatly indebted to Dr. Philipp Gerhardt, Chairman of the Department of Microbiology and Public Health, for his excellent research and academic leadership.

This study was supported by the National Institutes of Health Grant AI 05549 and the Michigan Agricultural Experiment Station.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The mechanism of neutralization of animal viral infectivity by antibody has been intensively studied. There has been no general agreement on the stability of the virus-antibody complex. The present investigation is concerned with the pH stability of infectious bronchitis virus-antibody complex and the interaction of the complex with chicken embryo kidney cells.

LITERATURE REVIEW

Infectious Bronchitis Virus

Infectious bronchitis virus (IBV), the causative agent of a respiratory disease of chickens (59), is 830 to 1,000 A^o in diameter (4, 49), contains ribonucleic acid (RNA) (1, 48), and has a lipoprotein coat with pear-shaped surface spikes (4, 49).

Some of the known physical properties of the virus are anisoelectric point at pH 4.05, anoptimal stability of infectivity at pH 7.8 (12),a density of 1.15 to 1.19 in sucrose (9, 15), and a sedimentation coefficient of 334 S (15). Viral protein contains sulfhydryl components as determined with p-chloromercuribenzoate (53).

Direct hemagglutination is not a characteristic of the virus. A noninfectious hemagglutinin can be isolated by anion exchange diethylaminoethylcellulose chromatography (5). It is 600 to 700 A^o in diameter and contains 51% protein, 34% lipid and trace amounts of carbohydrate and RNA.

Infectious bronchitis virus is sensitive to ether (1, 48, 66) and sodium deoxycholate (66).

The virus can be propagated in embryonating chicken eggs (12) and cell culture (16, 10, 11). The mode of replication has not been definitely established. Viral particles adsorb to the membrane of chicken embryo kidney cells (CEKC) within

30 minutes, are detectable in the nucleus within 7 hours (48) and in the cytoplasmic matrix within 12 hours (49). After 48 hours viral particles accumulate in the interstitial areas and are present on the surface of the cell membrane. Virus is released from the cell by budding and obtains an envelope from the cell membrane (49).

The frequency distribution of plaques formed in CEKC by the virus are according to the Poisson distribution. There is a linear relationship between the number of plaque forming units and the relative concentration (11).

Heating the virus at 100 C or treatment with ether releases antigens 1, 2, and 3. The antigens are noninfective and nonhemagglutinative (66).

Neutralization Reaction

Neutralization of virus by antibody is dependent on the concentration of antibody, temperature, pH, and ionic strength of the suspending medium (13, 44, 45). Viral survival, as a function of time, is a linear decrease over 99.9% of the reaction. The decrease is according to $-dc/dt = Kc$ where K is a proportionality constant, and $-dc/dt$ is the rate of change of the concentration of virus (c) with time (t) (44, 26). The K value may be used as a quantitative index of the effect of one variable on the virus-antibody reaction (13, 44, 45, 22).

A kinetic curve for anti-influenza-rabbit or fowl serum at 0 C had a lag phase and a phase of rapid exponential inactivation followed by a slower rate which was a reflection of the heterogeneity of the antibody used to inactivate the virus (38) or was due to a resistant fraction of virus (13, 44). At higher temperatures the lag phase was not pronounced (13, 57, 43). Nonneutralized adenovirus was coated with antibody or trapped by aggregates to an extent sufficient to change the density of the particle (36).

Three different types of combination between antibody and virus were detectable by electron microscopy: antibody in high concentration reacts with the virus through one of its active sites and radiates from the surface; in low concentration of antibody each antibody attaches to a single virus through sites at the ends of the molecule; at intermediate concentration of antibody, antibody molecules form bridges between adjacent viruses and cause aggregation (40).

Electron microscopy provides no simple explanation for the interaction of neutralized virus with the host cell. Viral infectivity of a neutralization mixture is influenced by the host system employed (67, 34, 6, 38, 69). Lafferty (39) has hypothesized that neutralization of a virus by any single antibody molecule depends on the electrostatic nature of the host cell surface.

Antibody molecules,γG, are cylindrical, 250 A$^{\circ}$ X 30 to 40 A$^{\circ}$ (23), and the active site is at the end (23, 2, 40). Antibody molecules to a single antigen may differ in molecular weight, electrophoretic mobility, and N-terminal amino acids (70).

Dissociation

Burnet et al. (8) concluded that the reaction of animal viruses with antibody was freely reversible. Fazekas de St Groth and Webster (19) substantiated this using noninactivated serum with influenza. Dulbecco et al. (13), Mandel (43), Kjellen (35), Lafferty (38), and Granoff (22) reported that neutralization was essentially irreversible at neutrality. If a state of equilibrium exist then dilution of the mixture should dissociate some of the combined reactants. Lafferty (38) reported recovery of infectivity by diluting after a brief period at 0 C. As the reaction time increased less infectivity was recovered on dilution. Dulbecco et al. (13) and Mandel (44, 45) reported that poliovirus-antibody complex did not dissociate upon dilution. Granoff (22) substantiated this using Newcastle disease virus (NDV). However, Dulbecco et al. (13) and Rubin and Franklin (57) reported a slight amount of dissociation (0.1 to 1.0%) of western equine encephalitis and NDV. Dilution of large amounts of poliovirus-antiserum mixtures resulted in 0.2% dissociation (44, 45).

Andrewes (3) and Gard (21) observed that prolonged reaction time stabilized virus-antibody complexes. Lafferty (38) proposed that a freely reversible complex is formed during the first stage of the reaction. In the second stage, a stable complex is formed when the free site of an antibody molecule projecting from the virus combines with another antigenic site on the same virus. Divalent antibody reacted with influenza virus (38) and with adenovirus (35, 36) to form a stable complex. Monovalent antibody formed an unstable complex that was dissociated by dilution (38, 36).

Kjellen (36) substantiated that secondary reactions are responsible for efficient inactivation of adenovirus. The animal virus-antibody complex formed by the secondary reaction were dissociated by low pH. The dissociation resulted in quantitative recovery of viral infectivity (43, 35, 52, 22). As the pH increased the degree of recovery decreased (45, 22). Granoff (22) recovered infectious virus by dissociating NDV-antibody complex at pH 1.3 and pH 12.1.

Poliovirus combined with minimal amounts of antibody and incubated for 120 days at 4 C can be recovered nearly quantitatively by dissociation at low pH. If large amounts of antibody were combined with virus no infectious virus could be recovered by dissociation at low pH (44). Hummeler and Ketler (28), Mandel (44), Lafferty (38), and Granoff (22) concluded that the infectivity of the virus was not irreversibly altered by combination with antibody.

Reactivated virus was reneutralized by the dissociated antibody (44) but at a slower rate than the initial neutralization (52). Kjellen (36) hypothesized that acidification caused new possible combinations between virus and antibody. Granoff (22) detected no difference in the rates of virus neutralization by untreated antiserum or antiserum acidified for 30 seconds, which indicated that antibody was not greatly altered.

Isliker (29) concluded that at low pH influenza virus and antibody do not interact because they both have a net positive charge. At neutral pH the virus and antibody bear opposite net charges and interact. Virus-antibody complexes did not dissociate in an antibody-free suspension at neutral pH (45, 22).

A nonavid antibody is one which is unable to form a stable complex with the virus surface. The complex formed by influenza virus and monovalent antibody dissociates when diluted at neutrality (38). Lafferty (38) interpreted the small amount of reversibility upon dilution at neutrality as indicative of monovalent, nonavid antibody which protected some viral particles from neutralization by avid antibody. Westaway (69) found 0.5 to 14% dissociation of neutralized Murray Valley encephalitis and West Nile. He concluded that heat labile virus inhibitors reacted with viral particles and modified the attachment of avid antibody.

Neutralized virus may be reactivated with fluorocarbon (32), ultrasonic vibration (39, 30), addition of inactivated virus (47, 13, 22), or by hypertonic saline solutions (63).

A virus-antibody complex was adsorbed to susceptible cells (27, 44, 22). However, adsorption did not occur when the virus was effectively covered by antibody (27, 57). The role of antibody in neutralization of viral infectivity is thought to be prevention of penetration (57, 45) or uncoating (22). A buffer of low pH dissociates the antibody from the cell-virus-antibody complex without simultaneously inducing detachment of virus from the cell. The virus is then free to infect the cell (43, 44, 22).

MATERIALS AND METHODS

Virus

The extracellular fluid of the 112th passage in chicken embryo kidney cells (CEKC) of the Beaudette culture of infectious bronchitis virus (IBV-42) was collected 60 hours post inoculation and stored at -60 C until used.

The CEKC extracellular fluid was centrifuged at 10,000 x G for 30 minutes at 5 C (Sorvall RC-2B, rotor SS-34) to remove extraneous materials. The supernatant fluid was centrifuged at 74,000 x G for 60 minutes at 5 C (Beckman model L, rotor 50). The pellet was suspended to original volume in phosphate buffered saline (PBS), pH 7.4 and stored at -60 C. This will be referred to as partially purified virus.

Antisera

Eight-month-old Single Comb White Leghorn cockerels were inoculated intranasally with 0.2 cc of IBV (16 x 10^5 chicken embryo infective doses $_{50}$). Six weeks later the chickens were reinoculated with 0.1 cc (8 x 10^5 chicken embryo infective doses $_{50}$) of virus. Three weeks after the second inoculation the chickens were fasted for 24 hours and exsanguinated. The sera were pooled, centrifuged, decanted, and stored at -20 C. Before being used, the antisera were inactivated at 56 C in a water bath for 30 minutes unless otherwise specified.

Immunoglobulin

Euglobulin was precipitated from the antisera by the method of Kendall (31). At 4 C 13 ml of saturated $(NH_4)_2SO_4$ was added slowly to 26 ml of the pooled antiserum. The mixture was stored for 24 hours at 4 C, centrifuged, and the precipitate was resuspended in PBS (0.15 M NaCl in 0.02 M phosphate), pH 7.0, to a total volume of 26 ml. The procedure was repeated three times. Sulfate ions were removed by dialysis against PBS for four days at 4 C. The PBS was changed daily. Pseudoglobulin was separated from the euglobulin by dialysis against distilled water for three days at 4 C with daily changes of distilled water. The water insoluble euglobulin was resuspended in PBS containing 0.3 M NaCl, pH 7.0, to a volume of 26 ml.

Sedimentation coefficients of the immunoglobulin were not determined.

Diluents

The diluent for the virus and virus-antibody mixtures was phosphate buffered saline (PBS) with 2% new born calf serum, pH 7.3. The diluent for antibody was Hanks' Balanced Salt solution, pH 7.0.

Determination of Protein

The Folin-Ciocalteu phenol reagent as modified by Lowry et al., (42) was used to determined 100 ug or more protein per ml. The micromodification (42) was used for less than

100 ug of protein per ml. Standards were prepared with 10 to 300 ug of bovine serum albumin (National Biochemical Corp., Cleveland, Ohio). A Beckman DB spectrophotometer was used.

Cell Culture

Primary chicken embryo kidney cell (CEKC) cultures, 10^7 cells per ml, were prepared from 17-to-18-day-old chicken embryos as described by Cunningham (10) and Cunningham and Spring (11).

The cultural medium consisted of medium 199 in Hank's Balanced Salt solution. This was supplemented with vitamins, amino acids, and L-glutamine (General Biochemicals) all having a final concentration of 1% in Eagle's basal medium. In addition 5% newborn calf serum (Grand Island Biological Company), 100 units penicillin, 0.1 mg dihydrostreptomycin and 100 units Mycostatin per ml were added, respectively. The medium was adjusted to neutrality with 10% saturated $NaHCO_3$. Plastic (Falcon) petri dishes, 60 x 15 mm, were seeded with 4 ml of the cell suspension per dish. Incubation was at 37 C, 85% relative humidity, and 8% CO_2 atmospheric changes per hour. A satisfactory monolayer of cells was formed after 48 hours.

Plaque Assay

Four cell cultures were used for each virus sample unless indicated otherwise. The cultural medium was decanted from 48-hour-old CEKC monolayers. The monolayers were washed with balanced salt solution and each was inoculated with 0.5 ml of a particular suspension of virus. The inoculated and control monolayers were incubated for 90 minutes. The fluid was decanted and the cells overlaid with 4 ml of the cultural medium containing 0.9% agar (Difco Noble). After the agar had solidified, the cultures were incubated 3 to 4 days. Neutral red, 0.1%, in PBS, pH 7.0, was added to the cultures. They were then incubated for 30 minutes at 37 C and 1 hour at 4 C. The number of plaques was counted and the infectivity titer calculated using the enumerative dose response. The infectivity titer was expressed in plaque forming units (PFU) per ml.

Effect of low and high pH on IBV infectivity

Virus, 4×10^4 PFU per ml, was mixed with equal volumes of Sorensen's glycine-HCl, 0.1 N HCl acid, Sorensen's glycine-NaOH, or 0.1 N NaOH. The final pH of the acidic and basic solutions was adjusted to pH 3.0 and 11.0, respectively, using 0.1 N HCl and 0.1 N NaOH. After incubation for 30 minutes at 4 C, the solution was diluted 10^{-2} and tested for virus.

Kinetics of Neutralization

The constant virus-decreasing serum neutralization test was based on the plaque reduction method (11). Equal volumes of virus, 5×10^4 PFU per ml, and anti-IBV-chicken serum were mixed and incubated at 37 C. At various time intervals, dilutions were made of each mixture and tested for virus. Virus control samples were processed in the same manner except that PBS was substituted for antiserum.

IBV - Antibody complex formation

A concentration of antibody that would neutralize 99.9% of the virus was used. Virus and antibody were always mixed in equal volume and incubated in water baths for 1 hour at 37 C and then for 21 hours at 4 C unless otherwise specified.

Effect of pH on the stability of IBV-antibody complex

Virus, 3 x 10^6 PFU per ml, was mixed with antiserum or immunoglobulin and incubated. Virus-antiserum mixtures were also incubated for 1 hour at 37 C and then for 14 days at 4, 23, 37 C. Test for dissociation of virus-antibody complex by high H^+ ion concentration were made in ice-cold Sorensen's glycine-HCl containing 0.1% gelatin at pH 1.5 to 4.0. Based on preliminary examination it was necessary to dilute the virus dissociated at 1.5 to 2.7 10^{-2}. In the case of pH 2.8 or greater dissociation was not as complete and only a 10^{-1} dilution was required. After one minute, the pH of the reaction mixture was brought to pH 7 by a 10^{-2} dilution into neutral PBS. The mixture was tested for virus immediately.

Effect of pH on neutralization potential of antiserum prior to combination with IBV

Antiserum diluted 1/80 was adjusted to pH 1.7 for 60 seconds at 4 C and then back to neutrality. Antisera previously acidified or untreated were mixed with virus, 1 x 10^5 PFU per ml, and incubated at 37 C. At various intervals a portion of each was diluted and tested for virus. For the virus controls PBS was substituted for the antiserum.

IBV-antibody complex in an antibody-free suspension

Virus and antibody were mixed and incubated as previously described. Virus-antibody complex was removed from the reaction mixture by centrifuging 24,000 x G for 1 hour at 5 C and then the sediment was resuspended to volume in PBS, pH 7.4.

Dissociation of IBV-antibody complex at pH 1.9 and reassociation

Virus, 3×10^6 PFU per ml, was mixed with antiserum and incubated. Virus-antibody complex was removed from the reaction mixture and diluted 10^{-1} in ice-cold Sorensen's glycine-HCl, pH 1.9. After 1 minute, two 0.5 ml samples were removed. One sample was added to 4.5 ml "PBS", pH 10.4. The final pH was 7.2. The other sample, the control, was diluted 10^{-3} to minimize the effect of antibody. Both mixtures were incubated at 37 C for 30 minutes. The control sample was tested without further dilution. The other sample was diluted 10^{-2} for compensatory dilution and then tested for virus.

Effect of pH on dissociation of IBV-antibody complex in an antibody-free suspension

Virus, 9×10^5 PFU per ml, was mixed with antiserum and incubated. Virus-antibody complex was removed from the reaction mixture and then acidified at pH 1.6, 1.7, or 1.9 as previously described.

Antibody dissociated at pH 1.7 and 2.7 from IBV-antibody complex

Partially purified virus, 2×10^5 PFU per ml, was mixed with immunoglobulin, 164 µg per ml, or antiserum both at a concentration that would neutralize 99.99% of the virus. The mixtures were incubated and then virus-antibody complex was removed from the reaction mixture. The amount of unneutralized virus was assayed and the sample was diluted 10^{-1} in Sorensen's glycine-HCl pH 1.7 or 2.7 without 0.1% gelatin. Test for dissociated virus was made as previously described. Antibody dissociated from virus was separated by centrifuging 74,000 x G for 30 minutes at 5 C. The pH of the supernatant fluid was adjusted to pH 7.0 and the amount of protein was determined. Undiluted samples were mixed with virus, 4×10^3 PFU per ml, and incubated at 37 C. At various intervals a portion of each mixture was diluted appropriately and tested for virus. Virus control samples were processed in the same manner except that PBS was substituted for the antiserum.

Effect of dilution on IBV-antibody complex

Virus, 4×10^5 PFU per ml, was mixed with non-inactivated antiserum, inactivated antiserum, or immunoglobulin and incubated. Virus mixed with non-inactivated antiserum was either incubated for 22 hours at 4 C or for 1 hour at 37 C and then 21 hours at 4 C. Threefold dilutions were made in PBS, pH 7.3. Undiluted and diluted samples were tested for dissociation immediately and after an additional 5 hours at 4 or 23 C.

Effect of dilution on IBV-antibody complex in an antibody-free suspension

Virus, 2×10^5 PFU per ml, was mixed with antiserum and incubated. Virus-antibody complex was removed from the reaction mixture. Undiluted samples and dilutions of 1/3 and 1/9 were tested for dissociation. The undiluted sample was also incubated at 4 C and tested for dissociation after 5 hours and 24 hours. To determine the amount of virus available for dissociation and to establish that neutralized virus had been removed from the reaction mixture, tests were made for acid-dissociable virus as previously described.

Effect of pH on cell-IBV-antibody complex

Virus, 3 x 10^5 PFU per ml, was mixed with antiserum at a concentration that would neutralize 99.99% of the virus and incubated. Virus control samples were processed in the same manner except that PBS was substituted for the antiserum. Appropriately diluted neutralized virus suspension and virus control suspension were inoculated onto CEKC monolayers. After adsorption, the monolayers were washed with PBS, pH 7.3. Ice-cold Sorensen's glycine-HCl, pH 1.7 or 2.6, was added to the monolayer for 10 seconds and the cells were washed twice with PBS, pH 7.3. For control, monolayers were washed with only PBS, pH 7.3. The total time from the addition of Sorensen's glycine-HCl onto a monolayer to the final washing was 30 seconds. The cell layers were overlaid immediately with cultural medium containing 0.9% Difco noble agar. For cell controls, uninoculated monolayers were treated in the same manner.

RESULTS

Effect of low and high pH on IBV infectivity

IBV was stabile for 30 minutes at pH 3.0 and labile at pH 11.0 (Table 1).

TABLE 1. Effect of low and high pH on IBV infectivity

Exposure to pH (30 min)	Reagent	Infectivity titer PFU/ml ($X10^4$)	Per cent decrease in infectivity
7.3	PBS (control)	4.08	0
3.0	Sorensen's glycine-HCl	3.92	3.92
3.0	0.1 N HCl	3.75	8.09
11.0	Sorensen's glycine-HCl	0.00999	99.76
11.0	0.1 N NaOH	0.2560	93.73

Kinetics of neutralization

The first part of the curve is linear to 99.9% neutralization followed by a decrease in the slope (Figure 1).

The neutralization rate constant (K) may be calculated from:

$$K = \frac{2.3\, D \log \frac{C_o}{C_t}}{t}$$

where C_o is the original concentration of virus, C_t is the concentration at time (t), and D is the final dilution of the antiserum (44, 22). For antiserum dilutions 1/80, 1/160, and 1/320, K equals 8376 min.$^{-1}$, 8364 min.$^{-1}$, and 1641 min.$^{-1}$ respectively.

Immunoglobulin

Immunoglobulin, 87 µg per ml, neutralized 1 x 10^6 PFU of virus per ml, 99.99%. The immunoglobulin was tested by disc electrophoresis and only a single broad band in the gamma globulin region was found.

Effect of pH on stability of IBV-antibody complex

The complex formed with antibodies in anti-IBV-chicken serum or immunoglobulin was dissociated over the range of pH 1.5 to 4.0 (Figure 2). The maximum amount of dissociation was at pH 1.7 (Table 2, Figure 2). The amount of dissociation varied directly with the H^+ ion concentration (Figure 3). At a pH lower than 1.7 the virus was inactivated by H^+ ions and less infectious virus was recovered. There was approximately 5 to 20% inactivation of the virus controls. Dissociation was demonstrable after 14 days at 4 C but not after 14 days at 23 or 37 C (Table 2).

Effect of pH on antiserum prior to combination with virus

Acidified and nonacidified antiserum had similar K values. The data were used for the 5-to-30-minute test period for comparative purposes (Table 3).

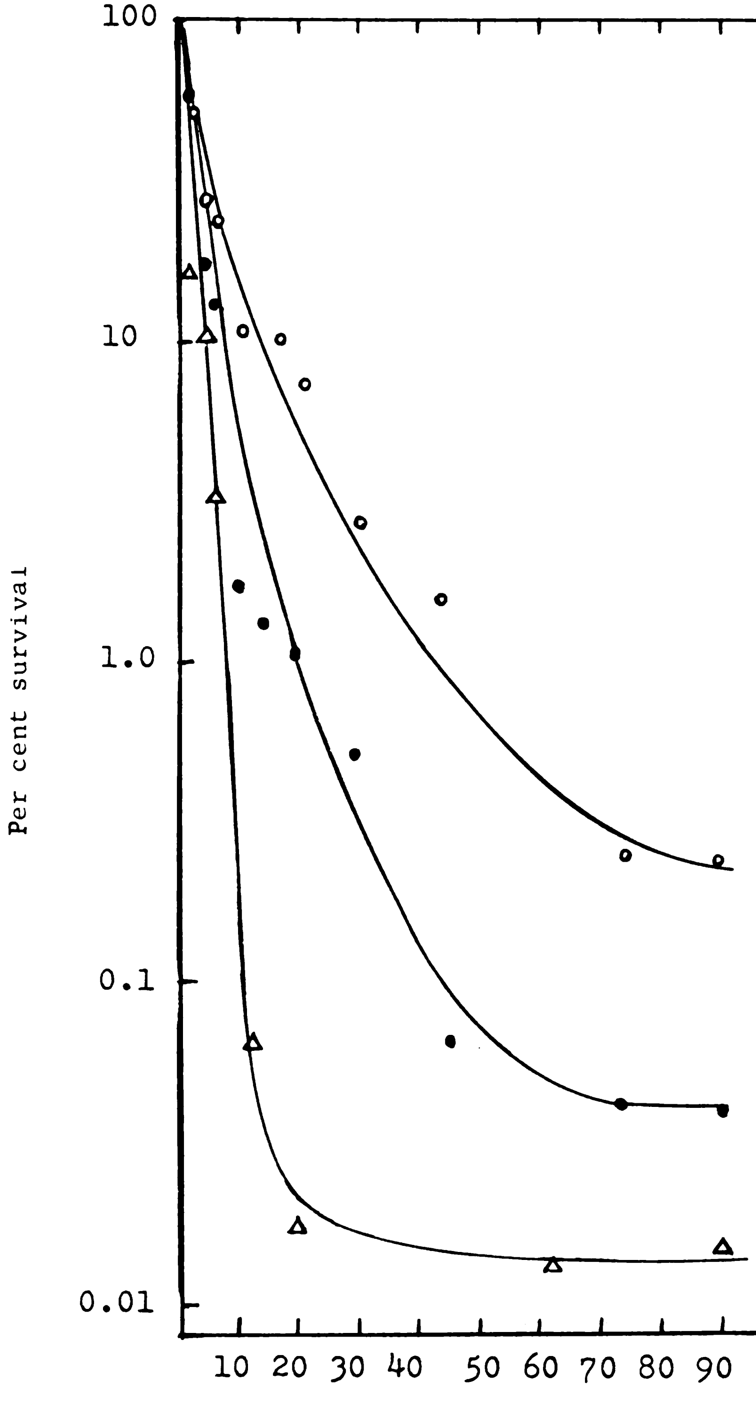

Figure 1. Kinetics of IBV neutralization by anti-IBV-chicken serum. Antiserum diluted 1/80 (△), 1/160 (●), 1/320(o).

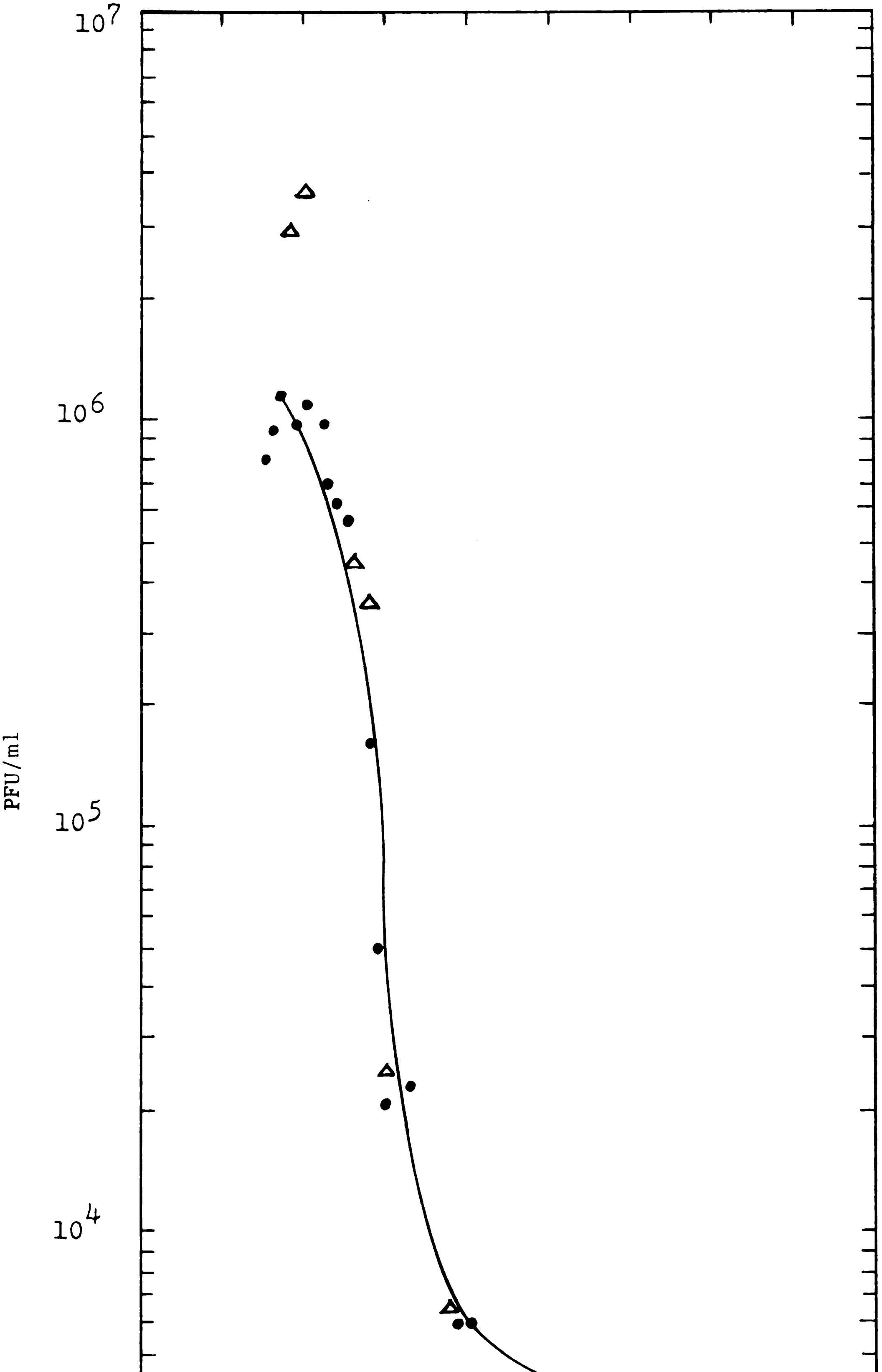

Figure 2. Dissociation of IBV-antibody complex. Virus antiserum mixture (●), virus-immunoglobulin mixture (△).

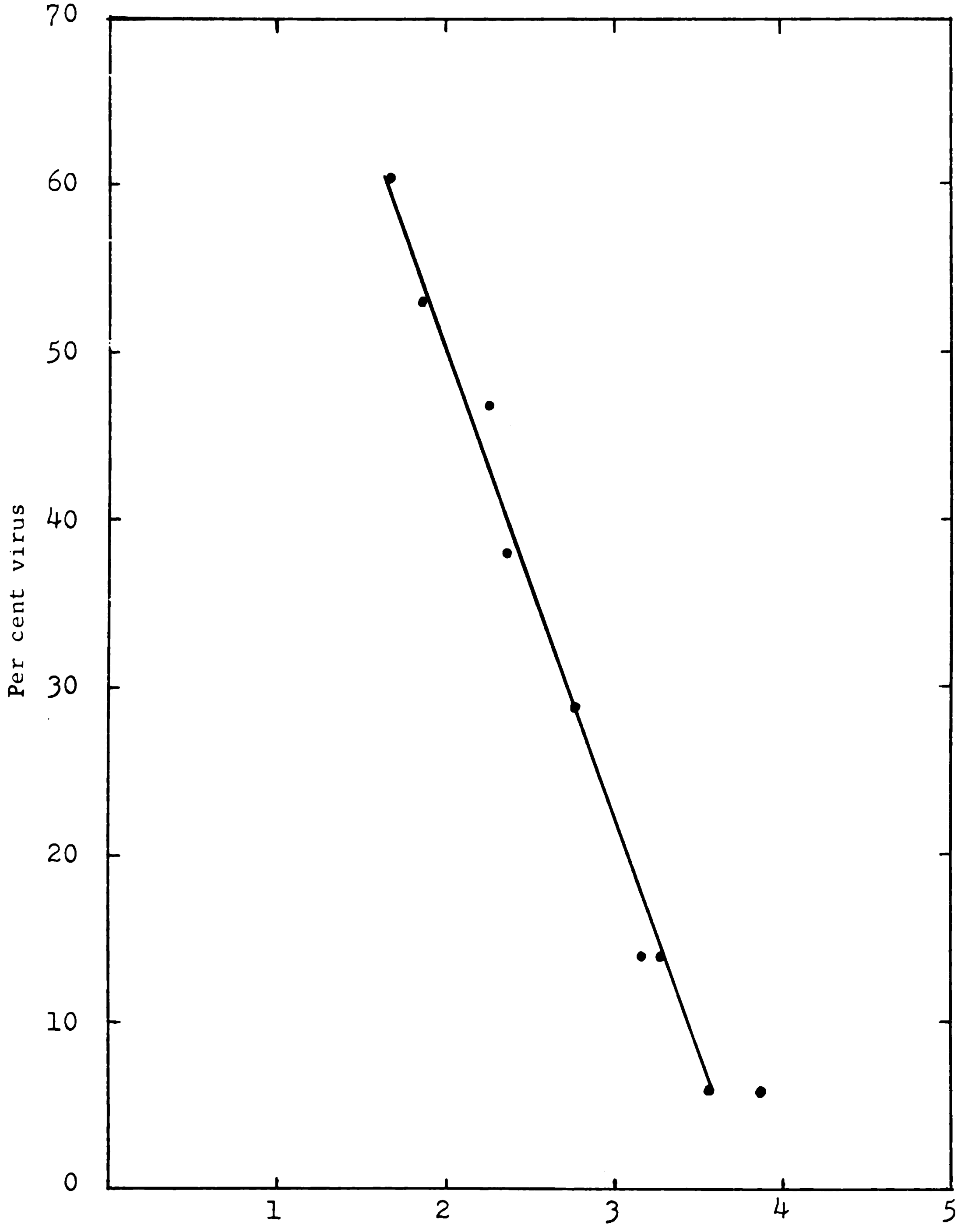

Figure 3. Per cent dissociation of IBV-antibody complex as a function of H^+ ion concentration.

TABLE 2. Effect of pH on stability of IBV-antibody complex

Neutralization	Dissociation pH	PFU/ml after dissociation (X10^6)	Per cent neutralized virus dissociated
7.3	1.7	1.14	60.50
(99.9% neutralized virus)	2.4	0.63	38.33
	3.2	0.018	13.95
	7.3	0.0037	0
Incubated 14 days at:			
4 C	1.7	0.28	23.25
23 or 37 C	1.7	0	0

TABLE 3. Effect of pH on neutralization potential of antiserum prior to combination with IBV

Antiserum treated for 1 min. at pH	Reagent	Incubation (min)	Infectivity titer (PFU/ml)	Neutralization rate constant (min.$^{-1}$)
7.3 (control)	PBS	5	6.04×10^3	
		30	4.00×10^1	1229
1.7	Sorensen's glycine-HCl	5	7.90×10^3	
		30	5.00×10^1	1163

Dissociation of IBV-antibody complex at pH 1.9 and reassociation

The virus-antibody complex, dissociated at pH 1.9, reassociated at neutrality (Table 4).

Effect of pH on dissociation of IBV-antibody complex in an antibody-free suspension

After centrifugation at 24,000 x G for 1 hour the IBV-antibody complex was sedimented. The complex dissociated at pH 1.6 to 1.9 but not at pH 7.3. Acidification increased the amount of infectious virus approximately 10^3 (Table 5).

Antibody dissociated at pH 1.7 and 2.7 from IBV-antibody complex

More antibody was dissociated at pH 1.7 than at pH 2.7 (Table 6). The "per cent dissociated virus" was decreased 1/10 when the H^+ ion concentration was decreased 1 pH unit. The µg of antibody obtained by acidification of virus-antibody complex was equal when the concentration of immunoglobulin or antiserum neutralized 99.99% of the virus. For antibody dissociated at pH 1.7 or 2.7, K equals 11.5 min.^{-1} and 7.03 min.^{-1}, respectively (Table 6).

Effect of dilution on IBV-antibody complex

When virus-inactivated antiserum mixtures were diluted, the average number of plaques was proportional to the dilution. A small amount of dissociation occurred after incubation of undiluted and diluted mixtures at either 4 or 23 C for 5 hours. There was no significant decrease in free virus in the original virus-inactivated antiserum mixture during incubation (Table 7).

TABLE 4. Dissociation of IBV-antibody complex at pH 1.9 and reassociation

Virus U.neutralized PFU/ml	Virus PFU/ml	Virus dissociated PFU/ml	Per Cent dissociated virus	Virus not Reneutralized PFU/ml	Virus reneutralized PFU/ml	Per cent virus reneutralized in 30 min. at 37 C
1.922×10^3	5.99×10^5	5.97078×10^5	99.68	1.976×10^5	3.99478×10^5	66.90

If virus was mixed with noninactivated antiserum and incubated at 4 C for 22 hours, a 1/9 dilution of the mixture resulted in dissociation (Table 8). Results were similar with undiluted and diluted mixtures after 5 hours at 4 or 23 C (Table 8). There was no significant decrease in free virus in the undiluted samples.

If virus was mixed with noninactivated antiserum and incubated at 37 C for 1 hour followed by 21 hours at 4 C, a 1/9 dilution of the mixture resulted in only 0.06% dissociation.

A small amount of dissociation occurred by 1/9 dilution of virus-immunoglobulin mixture (Table 7).

Effect of dilution of IBV-antibody complex in an antibody free suspension

Dissociation of the IBV-antibody complex does not occur in an antibody-free suspension at 4 C even when 1 x 10^5 PFU per ml are available for dissociation (Table 9).

When the complex was diluted, the average number of plaques was proportional to the dilution (Table 10).

Effect of pH on cell-IBV-antibody complex

Specific attachment of the virus-antibody complex to the cell occurred within 90 minutes. Washing the monolayer of cells with PBS, pH 7.3, after attachment of the virus-antibody complex, or virus alone, was without effect. However, when Sorensen's glycine-HCl, pH 1.7 or 2.6, was used for 10 seconds as a wash, antibody dissociated from the complex but there was no effect on the virus attached to the cell (Table 11). The per cent dissociation depended upon the H^+ ion concentration.

TABLE 5. Effect of pH on dissociation of IBV-antibody complex in an antibody-free suspension

pH	Virus unneutralized PFU/ml	Virus PFU/ml	Virus dissociated PFU/ml	Per cent dissociated virus PFU/ml
1.6	3.0×10^2	2.18×10^5	2.177×10^5	99.86
1.7	"	2.05×10^5	2.047×10^5	93.89
1.9	"	1.346×10^5	1.343×10^5	61.61
7.3	"	3.00×10^2	0	0

TABLE 6. Antibody dissociated at pH 1.7 and 2.7 from IBV-antibody complex

Virus neutralized by immunoglobulin								
					Antibody diluted 1/10			
pH	Virus unneutralized PFU/ml	Virus PFU/ml	Virus dissociated PFU/ml	Per cent dissociated virus	μg antibody dissociated	Incubation (min.)	Infectivity PFU/ml	K (min.$^{-1}$)
1.7	3.2×10^1	1.0×10^5	9.9968×10^4	99.68	145	60 (virus control)	2.40×10^4	
						5	7.60×10^3	
						60	2.76×10^3	11.5
2.7	"	9.9×10^3	9.9680×10^3	9.97	~ 30*	5	4.20×10^3	
						60	2.50×10^3	7.0
Virus neutralized by antiserum								
1.7	0.9×10^1	1.0×10^5	9.9991×10^4	99.99	135			
2.7	"	9.909×10^3	9.9×10^3	9.99	~ 20*			

*$\sim$ = approximately

TABLE 7. Effect of dilution on IBV-antibody complex. I. Inactivated antiserum

	Hr. after dilution	Dilution employed	Number of cultures	$\bar{X}$ PFU[a]	b σ	Expected $\bar{X}$ PFU	Per cent dissoc. based on conversion to PFU/ml[c]
Virus + inactivated antiserum	Zero	Undiluted	11	14.63	5.12	-	
		1/3	11	5.72	1.74	4.88	
		1/9	10	2.50	1.27	1.62	0
	5 hr. @ 4 C	Undiluted	15	18.20	6.83	-	
		1/3	16	14.19	3.29	6.06	
		1/9	16	7.06	2.08	2.02	0.06
	5 hr. @ 23 C	Undiluted	14	14.36	6.33	-	
		1/3	16	8.56	2.65	4.78	
		1/9	16	5.56	3.64	1.60	0.04
Virus + immunoglobulin	Zero	Undiluted	4	21.50	1.73	-	
		1/3	4	18.50	5.06	7.17	
		1/9	4	7.25	3.30	2.70	0.04

a Average number of plaque forming units; b ± standard deviation.

c These data are derived from the original mixture when 99.9% of the virus were neutralized.

TABLE 8. Effect of dilution on IBV-antibody complex
II. Noninactivated antiserum

	Hr. after dilution	Dilution employed	Number of cultures	$\bar{X}$ PFU[a]	b σ	Expected $\bar{X}$ PFU	Per cent dissoc. based on conversion to PFU/ml[c]
Virus + noninactivated antiserum	Zero	Undiluted	4	51.0	6.74	-	
		1/3	4	43.0	7.64	17.00	
		1/9	4	40.0	5.92	5.66	0.31
	5 hr. @ 4 C	Undiluted	4	53.5	7.78	-	
		1/3	4	47.0	6.82	17.50	
		1/9	4	50.0	10.62	5.94	0.40
	5 hr. @ 23 C	Undiluted	4	40.0	2.24	-	
		1/3	4	28.0	2.21	13.33	
		1/9	4	28.0	5.19	4.44	0.21

a Average number of plaque forming units; b ± standard deviation.

c These data are derived from the original mixture when 99.9% of the virus were neutralized.

TABLE 9. Effect of incubation on IBV-antibody complex in an antibody-free suspension

	Hours	Number of cultures	$\bar{X}$ PFU[a]	σ[b]	Virus available for dissociation PFU/ml[c]
Virus + inactivated antiserum	Zero	7	70.14	11.19	1.24×10^5
	5	8	63.25	5.44	1.01×10^5
	24	8	17.38	6.39	5.50×10^4

[a] Average number of plaque forming units; b ± standard deviation.

[c] Determined by acid-dissociation.

TABLE 10. Effect of dilution on IBV-antibody complex in an antibody-free suspension

	Dilution employed	Number of cultures	$\bar{X}$ PFU[a]	σ[b]	Expected X PFU	Per cent dissociation
Virus + inactivated antiserum	Undiluted	7	70.14	11.19	--	
	1/3	8	22.87	7.30	23.38	
	1/9	8	8.12	3.24	7.79	0

[a] Average number of plaque forming units; [b] ± standard deviation.

TABLE 11. Effect of pH on dissociation of IBV-antibody complex after adsorption to cells

Inoculum	pH	Infectivity PFU/ml ($X10^4$)	Per cent dissociation
Virus + buffer	7.3	33.0	-
	1.7	35.0	-
	2.6	38.5	-
Virus + antiserum	7.3	0.009	-
	1.7	7.860	22.47
	2.6	1.14	2.96

DISCUSSION

The curve for the IBV-antibody interaction follows first-order kinetics with the reaction rate dependent upon time, antibody concentration, and temperature and is similar to the results reported by Dulbecco (13), Mandel (44), and Granoff (22) with other virus-antibody systems.

Acid stability (71) is the only criterion studied by which IBV differs from the general characteristics of myxoviruses.

At neutrality the IBV-antibody complex is stable. Dissociation occurs within 30 seconds at pH 4.0 or lower and confirms Granoff's (22) report that the amount of dissociation is dependent upon the H^+ ion concentration.

Mandel (44) and Granoff (22) both concluded that dissociation is not due to antibody denaturation based on the results, respectively; 1) dissociated virus and antibody could reassociate at neutrality, 2) acidification of antiserum for 30 seconds has no effect upon the neutralization rate constant. It is unlikely, however, that a protein would not be denatured at low pH. It is possible that antibody changes configuration at low pH as does albumin (60) and returns to the original configuration at neutrality. Since acidification of virus and antibody below the isoelectric point would induce a net positive charge, the mechanism of dissociation could be due to electrostatic repulsion between the chains of the antibody. This would alter the active sites with an accompanying reduction of van der Waals' attraction and the sites would no longer be complementary. A decrease of H^+ ions results in less positive charge induction and as a result a smaller amount of dissociation.

Since IBV was inactivated at a high pH, dissociation of virus-antiserum or immunoglobulin mixtures could only be proven in the low pH range where the virus is stabile. The inability to detect 100% dissociation may be due to reassociation, lack of complete dissociation, or irreversible alteration of the virus.

Dissociation of IBV-antibody complex after 14 days at 4 C with recovery of infectious virus emphasizes that antibody does not permanently alter the infectious capacity of virus. This is in complete agreement with the reports of Mandel (44) and Granoff (22) using poliovirus and NDV, respectively, under similar conditions. It was not possible, however, to detect dissociation after 14 days at 23 or 37 C. The virus is inactivated at these temperatures (11).

Antibody obtained from dissociated IBV-antibody complex will neutralize virus. The slow rate of neutralization could be due to some denaturation or simply the result of decreased protein concentration. These results favor the conclusion that antibody is denatured, but not irreversibly altered at low pH.

Interaction between NDV and antibody has been reported by Burnet (8) to be completely reversible by dilution. This report was refuted by Rubin and Franklin (57). Likewise, Mandel (44) and Granoff (22) concluded that the interaction is essentially irreversible by dilution. However, Mandel (45) reported 0.2% dissociation, when poliovirus and antibody were mixed in high concentration. When high concentration of IBV and antibody were mixed, dilution of the complex does not cause dissociation. If the complex is diluted and then incubated, dissociation, 0.06%, occurs. Dissociation is considered a valid conclusion since there was no decrease in free virus in the undiluted mixtures during incubation. If, however, the antiserum is not inactivated and the reactants are incubated at 4 C, 0.3% dissociation will occur. Dissociation was detected when the average number of plaques was not proportional to the dilution within the limits of the standard deviation.

It is possible that the presence of heat labile nonspecific protein in noninactivated antiserum hampers the complete mechanism of neutralization and as a result causes a small amount dissociation by dilution.

The IBV-antibody complex separated from the reaction mixture by centrifugation was stabile and no dissociation occurred after incubation at 4 C or by a 1/9 dilution. The complex dissociated only at low pH. These data confirm the previous findings that virus-antibody complex is essentially irreversible at neutrality.

Anti-IBV-antisera were prepared in chickens. In contrast, Mandel (44) and Granoff (22) obtained antisera from animals which are not the normal host for the virus used.

The small amount of dissociation, 0.04%, of virus neutralized by immunoglobulin may have been the result of a slight amount of alteration of the active sites when the globulin was fractionated from antiserum. Lack of precise complementarity could result in weak bonding between the virus and the antibody.

The data presented confirm that a virus-antibody complex, and specifically one with IBV, adsorbs to susceptible cells. Antibody can be dissociated from the complex without simultaneously inducing detachment of infectious virus from the cell. The mechanism of the effect of antibody at the cell membrane is not understood. Mandel (44) hypothesized that antibody blocks penetration of the cell by simple steric hindrance. Likewise, it may be possible that antibody affects cell membrane envagination. Granoff (22) proposed that antibody prevents uncoating of viral protein.

The experiments described in this investigation and the results obtained with other virus-host systems substantiate that virus-antibody complex dissociates at low pH, virus is not irretrievably deprived of infectiousness by neutralizing antibody, and virus-antibody complex is essentially irreversible at neutrality.

SUMMARY

1. Infectious bronchitis virus (IBV) is acid stabile and base labile.
2. Virus neutralized by antibodies in either antiserum or immunoglobulin can be dissociated at pH 4.0 or lower and infectious virus recovered. A maximum of 60% dissociation of IBV-antibody complex occurred at pH 1.7. The amount of dissociation depends upon the H^+ ion concentration.
3. After 14 days at 4 C, the complex can be dissociated at pH 1.7 and infectious virus recovered. Antibody does not permanently alter the infectious capacity of the virus.
4. Antibody acidified for one minute at pH 1.7 was not irreversibly altered. Return to neutrality renatured the molecule. Acidified and nonacidified antiserum had similar neutralization rate constants. Infectious bronchitis virus and antibody dissociated at pH 1.9 reassociated at neutrality.
5. The µg of antibody dissociated from IBV was related to the H^+ ion concentration. Dissociated antibody retained its capacity to neutralize virus.
6. Dissociation did not occur when IBV-inactivated antiserum mixtures were diluted at neutrality. An extremely small amount of dissociation did occur when the complex was

diluted and then incubated at either 4 or 23 C for 5 hours. The largest amount of dissociation occurred when IBV-noninactivated antiserum mixtures were diluted at neutrality. The presence of heat labile nonspecific protein in the serum may be related to the amount of dissociation by dilution.

7. The IBV-antibody complex in an antibody-free suspension was stable and did not dissociate when incubated at 4 C or when diluted 1/9.
8. The IBV-antibody complex adsorbed to a chicken embryo kidney cell. Washing for 10 seconds at pH 1.7 dissociated antibody from the virus without detachment of virus from the cell. The virus was then capable of infecting the cell.

LITERATURE CITED

1. Akers, T. G. 1963. Some cytochemical studiesoof the multiplication of infectious bronchitis virus in chicken embryo kidney cells. Ph. D. thesis, Michigan State University, East Lansing.

2. Almeida, J., B. Cinader, and A. Hawatson. 1963. The structure of antigen-antibody complexes. J. Exptl. Med. 118:327-340.

3. Andrewes, C. H. 1930. Antivaccinal serum. III. Evidence for slow union with virus *in vitro*. J. Pathol. Bacteriol. 33:265-272.

4. Berry, D. M., J. G. Cruickshank, H. P. Chu, and R. J. H. Wells. 1964. The structure of infectious bronchitis virus. Virology 23:403-407.

5. Biswal, N. 1965. The hemagglutination of infectious bronchitis virus. Ph. D. thesis, Michigan State University, East Lansing.

6. Bradish, C. J., J. O. Farley, and E. N. Ferrier. 1962 Studies on the nature of the neutralization reaction and the competition for neutralizing antibody between components of the virus system of foot-and-mouth disease. Virology 18:378-400.

7. Brunner, K. T., and R. Ward. 1959. Differences in stability of antigen-antibody complexes formed with poliovirus and acute and convalescent phase human sera. J. Immunol. 83:405-410.

8. Burnet, F. M., E. V. Keogh, and D. Lush. 1937. Immunological reactions of filterable viruses. Australian J. Exptl. Biol. Med. Sci. Suppl. 15:231-368.

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

10. Cunningham, C. H. 1963. "A laboratory guide in virology" 5th ed., Burgess Publishing Co., Minneapolis.

11. Cunningham, C. H., and M. P. Spring. 1965. Some studies of infectious bronchitis virus in cell culture. Avian Dis. 9:182-192.

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

13. Dulbecco, R., M. Vogt, and A. G. R. Strickland. 1956. A study of the basic aspects of neutralization of two animal viruses, western equine encephalitis virus and poliomyelitis virus. Virology 2:162-205.

14. Edelman, G. M., and M. D. Poulik. 1961. Studies on structural units of the gamma-globulins. J. Exptl. Med. 113:861-884.

15. Ellis, L. F. 1965. Centrifugation studies of infectious bronchitis virus. Ph. D. thesis, Michigan State University, East Lansing.

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

17. Fazekas De St. Groth, S., and R. G. Webster. 1963. The neutralization of animal viruses. III. Equilibrium conditions in the influenza virus-antibody system. J. Immunol. 90:140-150.

18. Fazekas De St. Groth, S., and A. F. Reid. 1958. The neutralization of animal viruses. II. A critical comparison of hypotheses. J. Immunol. 80:225-235.

19. Fazekas De St. Groth, S., and R. G. Webster. 1963. The neutralization of animal viruses. III. Equilibrium conditions in the influenza virus-antibody system. J. Immunol. 90:140-150.

20. Fazekas De St. Groth, S., and R. G. Webster. 1963. The neutralization of animal viruses. IV. Parameters of the influenza virus-antibody system. J. Immunol. 90:151-164.

21. Gard, S. 1955. Neutralization of theilers virus. Acta. Pathol. Microbiol. Scand. 37:21-30.

22. Granoff, A. 1965. The interaction of newcastle disease virus and neutralizing antibody. Virology 25:38-47.

23. Hall, C. E., A. Nisonoff, and H. S. Slayter. 1959. Electron microscopic observations of rabbit antibodies. J. Biophys. Biochem. Cytol. 6:407-413.

24. Hamparian, V. V., R. M. Hilleman, and A. Ketler. 1963. Contributions to characterization and classification of animal viruses. Proc. Soc. Exp. Biol. Med. 112:1040-1050.

25. Hashimoto, N., and A. M. Prince. 1963. Kinetic studies on the neutralization reaction between Japanese encephalitis virus and antiserum. Virology 19: 261-272.

26. Hiatt, C. W. 1964. Kinetics of the inactivation of viruses. Bact. Rev. 28:150-152.

27. Hultin, J. V., and A. P. McKee. 1952. Fixation of neutralized influenza virus by susceptible cells. J. Bacteriol. 63:437-447.

28. Hummeler, K., and A. Ketler. 1958. Dissociation of poliomyelitis virus from neutralizing antibody. Virology 6:297-299.

29. Isliker, H. 1964. Purification of virus antibodies, in "Immunological Methods," (J. F. Ackroyd, editor). F. A. Davis Company, Philadelphia, Pa. 137-139.

30. Keller, R. 1965. Reactivation by physical means of antibody-neutralized poliovirus. J. Immunol. 94: 143-149.

31. Kendall, F. E. 1938. Studies on serum proteins. I. Identification of a single serum globulin by immunological means and its distribution in the sera of normal individuals. J. Clin. Invest. 16:921-931.

32. Ketler, A., Y. Hinuma, and K. Hummeler. 1961. Dissociation of infective poliomyelitis virus from neutralizing antibody by fluorocarbon. J. Immunol. 86:22-27.

33. Kjellen, L. E. 1957. Studies on in vitro neutralization of adenoviruses. Arch Ges. Vriusforsch. 7:307-322.

34. Kjellen, L. E., and R. W. Schlesinger. 1959. Influence of host cell on residual infectivity of neutralized vesicular stomatitis virus. Virology 7:236-239.

35. Kjellen, L. E. 1962. Studies on the interactions of adenovirus, antibody and host cells in vitro. Virology 18:448-456.

36. Kjellen, L. E. 1965. Stabilization in acid of mercaptoethanol-reduced adenovirus antibody by combination with virus. Virology 27:580-588.

37. Kjellen, L. E. 1965. Density gradient centrifugation of adenovirus-gammaglobulin complexes. Arch. Ges. Virusforch. 17:398-408.

38. Lafferty, K. J. 1963. The interaction between virus and antibody. I. Kinetic studies. Virology 21: 61-75.

39. Lafferty, K. J. 1963. The interaction between virus and antibody. II. Mechanism of the reaction. Virology 21:76-90.

40. Lafferty, K. J., and S. Oertelis. 1963. The interaction between virus and antibody. III. Examination of virus-antibody complexes with the electron microscope. Virology 21:91-99.

41. Lafferty, K. J. 1965. The relationship between molecular charge and the biological activity of antibody directed against rabbitpox virus. Virology 25:591-602.

42. Lowry, O. H., N. J. Rosebrough, L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.

43. Mandel, B. 1958. Studies on the interactions of poliomyelitis virus, antibody, and host cells in a tissue culture system. Virology 6:424-447.

44. Mandel, B. 1960. Neutralization of viral infectivity: Characterization of the virus-antibody complex including association, dissociation, and host-cell interaction. Ann. N. Y. Acad. Sci. 83:515-527.

45. Mandel, B. 1961. Reversibility of the reaction between poliovirus and neutralizing antibody of rabbit origin. Virology 14:316-328.

46. Mandel, B. 1962. Early stages of virus-cell interaction as studied by using antibody. Cold Spring Harbor Symp. Quant. Biol. 27:123-131.

47. McKee, A. P., and W. M. Hale. 1946. Reactivation of overneutralized mixtures of influenza virus and antibody. J. Immunol. 54:233-243.

48. Mohanty, S. D., N. M. Devolt, and J. B. Faber. 1964. A fluorescent antibody study of infectious bronchitis virus. Poultry Sci. 43:179-189.

49. Nazerian, K. 1965. Electron microscopic studies of infectious bronchitis virus. Ph. D. thesis, Michigan State University, East Lansing.

50. Pauling, L. 1945. Molecular structure and intermolecular forces, in "The specificity of serological reactions," (K. Landsteiner, editor), Harvard Univ. Press. 275-293.

51. Philipson, L. 1966. Interaction between poliovirus and immunoglobulins. II. Basic aspects of virus-antibody interaction. Virology 28:35-46.

52. Pinheiro, F., and G. D. Hsiung. 1963. A study on dissociation of coxsackie B_4 virus-antibody complex. Virology 20:457-463.

53. Powers, C. D. 1965. Inactivation of infectious bronchitis virus infectivity by para-chloromercuribenzoate. M. S. thesis, Michigan State University, East Lansing.

54. Rappaport, I. 1959. The reversibility of the reaction between rabbit antibody and tobacco mosaic virus. J. Immunol. 82:526-534.

55. Rappaport, I. 1961. Acid dissociation of aggregates of antibody and tobacco mosaic virus with recovery of infectious virus. Biochim. et. Biophys. Acta. 47:206-208.

56. Reithel, F. J. 1963. The dissociation and association of protein structures, in "Advances in Protein Chemistry," (C. B. Anfinsen, M. L. Anson, and J. T. Edsall, editors), Academic Press, N. Y. 18:177, 197.

57. Rubin, H., and R. M. Franklin. 1957. On the mechanism of newcastle disease virus neutralization by immune serum. Virology 3:84-95.

58. Rubin, H. 1957. Interactions between newcastle disease virus, antibody, and cell. Virology 4:533-562.

59. Schalk, A. F., and M. Cm Hawn. 1931. An apparently new respiratory disease of chicks. J. Am. Vet. Med. Assn. 78:413-422.

60. Slayter, E. M. 1965. An electron microscopy study of the conformational change in bovine serum albumin at low pH. J. Mol. Biol. 14:443-452.

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

62. Svehag, S. E. 1963. Reactivation of neutralized virus by fluorocarbon: Mechanism of action and demonstration of reduced reactivability with time of virus-antibody interaction. Virology 21:174-182.

63. Svehag, S. E. 1965. The dissociability of different poliovirus-antibody complexes as tested by hypertonic salt solutions. Archiv fur die Gesamte Virusforschung. 17:504.

64. Szenberg, A., P. Lind, and K. Clarke. 1965. IgG and IgM antibodies in Fowl serum. Aust. J. Exptl. Bio. and Med. Sci. 43:451-454.

65. Taylor, R. M. 1941. Reactivation of neutralized influenza A virus by dilution. J. Immunol. 40: 373-381.

66. Tevethia, S. S. 1964. Studies of the antigens of infectious bronchitis virus. Ph. D. thesis, Michigan State University, East Lansing.

67. Tyrrell, D. A. J., and F. L. Horsfall. 1953. Neutralization of viruses by homologous immune serum. I. Quantitative studies on factors which affect the neutralization reaction with Newcastle disease, influenza A, and bacterial virus T_3. J. Exptl. Med. 97:845-861.

68. Watson, J. D. 1965. The importance of weak chemical interaction in "Molecular Biology of the Gene," W. A. Benjamin, Inc. N. Y., 114.

69. Westaway, E. G. 1965. The neutralization of arboviruses. I. Neutralization in homologous virus-serum mixtures with two group B arboviruses. Virology 26:517-527.

70. Weil, A. J., A. I. Parfentiev, and K. L. Bowman. 1938. Antibodies and their characterization. J. Immunol. 35:399. Cited by E. Kabat and M. Mayer (1961) in "Experimental Immunochemistry," Charles C. Thomas, Publisher, Springfield, Ill., 342.

71. Wilner, B. I. 1965. A classification of the major groups of human and lower animal viruses. 3rd ed. Burgess Publishing Company, Minneapolis, Minn.