INTERACTION OF NONNEUTRALIZED AND NEUTRALIZED AVIAN INFECTIOUS BRONCHITIS VIRUS WITH THE CHICKEN EMBRYO KIDNEY CELL

I. ENTRY INTO THE CELL
II. VIRAL DEGRADATION

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY

Mark F. Stinski

1969

This is to certify that the

thesis entitled
Interaction of nonneutralized and neutralized avian infectious bronchitis virus with the chicken embryo kidney cell.

presented by
Mark F. Stinski

has been accepted towards fulfillment
of the requirements for

Ph. D. degree in Microbiology

C. H. Cunningham

Major professor

Date 17 April 1969

ABSTRACT

INTERACTION OF NONNEUTRALIZED AND NEUTRALIZED AVIAN INFECTIOUS BRONCHITIS VIRUS WITH THE CHICKEN EMBRYO KIDNEY CELL

By

Mark F. Stinski

The rate of entry of avian infectious bronchitis virus (IBV) without and with antibody into chicken embryo kidney cells (CEKC) was determined. The virus absorbs to CEKC at 4° but it does not enter the cell. When the temperature was raised to 25 or 37°, entry of virus without and with antibody was linear. The kinetics of entry of virus without antibody was faster than for virus with antibody. Therefore, antibody affected either the rate or the mechanism of entry. Antibody had no effect on viral elution from the cell.

Entry of virus without antibody was measured at various intervals of time by neutralizing the extracellular virus. Because the intracellular virus could not be neutralized, it was infectious and therefore could be enumerated by the plaque method. Entry of virus with antibody was measured by determining the amount of extracellular-neutralized virus at various intervals of time. This was done by the use of a buffer at pH 2.0 which dissociated the antibody from the virus

with accompanying reactivation of viral infectivity and thus, this reaction could also be enumerated by the plaque method. Acid treatment did not injure the cell, dissociate virus from the cell, or interfere with the replication of the virus. Intercellular neutralized virus was not affected by the acid and consequently was not reactivated.

To further study the effect of antibody on the virus-cell interaction, IBV was labeled with ^{32}P. There was an identical relationship between adsorption of infectious virus and radioactivity of virus purified by centrifugation and chromatography.

Analysis of the extracellular fluid and the cells for degraded viral material indicated that virus without antibody was degraded at the same rate as virus with antibody. After 2 hrs at 37^{o} in cells with nonneutralized virus, 12% of the radioactivity was acid-insoluble, RNase sensitive material. However, little or none of this material was detected in cells with neutralized virus. Since antibody reacted with the viral projections and not with the envelope, it is hypothesized that attachment of antibody to these projections influences degradation of the virion and viral replication is prevented.

INTERACTION OF NONNEUTRALIZED AND NEUTRALIZED AVIAN INFECTIOUS BRONCHITIS VIRUS WITH THE CHICKEN EMBRYO KIDNEY CELL

I. Entry into the Cell
II. Viral Degradation

By

Mark F. Stinski

A THESIS

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

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1969

DEDICATION

I dedicate this thesis to a person who has given me her love and trust. She shares with me a common goal to which she unselfishly contributes. My accomplishments are a reflection of Mary Ellen Stinski's dedication and love. Truly she is a gift from God.

ACKNOWLEDGEMENTS

I wish to acknowledge the graduate educational opportunities and scientific challenges provided by the Department of Microbiology at Michigan State University. I appreciate the scholastic and research guidance provided by Dr. Charles H. Cunningham and Dr. Philipp Gerhardt. The financial assistance of a National Institute of Health traineeship obtained with the help of Dr. Harold Sadoff was greatly appreciated. St. John's Student Center provided moral and spiritual inspiration that strengthen my faith. I would like to thank Dr. Bruce Lacey, Martha Spring, and Lorell Angelety for their friendship, scientific consultation, and encouragement.

TABLE OF CONTENTS

TABLE OF CONTENTS - Continued

LIST OF TABLES

LIST OF FIGURES

LITERATURE REVIEW

Avian infectious bronchitis virus

Avian infectious bronchitis virus (IBV) has a ribonucleic acid core (2,42). The capsid is surrounded by a lipoprotein envelope that has pear-shaped projections (peplomers) 200 A^{o} long (4,6,47). Avian infectious bronchitis virus is pleomorphic and from 800 to 1000 A^{o} in diameter. Although IBV is similar to the myxovirus group in nucleic acid type, ether lability, and size (41), it differs from myxo- and paramyxoviruses in that the nucleoid is not distinct, the shell is poorly defined, and budding does not occur at the cell membrane (41,48).

Embryonating chicken eggs (12) and cell culture (10,11, 18,34) are used to propagate the virus. According to Lukert (34), adsorption of IBV to cells is an inefficient process since large amounts of virus remain unadsorbed. Chicken embryo kidney cells are more efficient than a number of other cells (34). The amount of absorption on chicken embryo kidney cells is similar at 4^{o} and "ambient" temperature. However, at 37^{o} adsorption is 3 to 5 times more efficient (34).

Replication is intracytoplasmic (5,41,48) and the virus buds into cisternae or vesicles, with an incorporation of host cell material as its outer coat (41,48). Berry and Almeida (6) proposed that the viral envelope component is closely related to chick embryo fibroblast membrane since antibody from homotypic antiserum reacted with the projections but not the envelope. In contrast, antibody from heterotypic antiserum reacted with both the projections and the envelope. Heterotypic antiserum prepared against only chick embryo fibroblasts membrane reacted with the viral envelope (6).

Interaction of neutralized virus with the host cell

Antibody neutralized virus adsorbs to susceptible cells without inducing viral replication (19,30,35,36,54). Factors that influence the adsorption of neutralized virus, such as poliovirus, are the molecular species of antibody, period of incubation, and the concentration of antibody (37). More virus adsorbs to cells when neutralized with IgG antibody than with IgM antibody. Adsorption is increased by long incubation periods and low concentrations of antibody (37).

Interpretations on the nature of the interaction between neutralized virus and the host cell are that antibody initiates viral elution from the cell (53), antibody blocks viral entry (35) neutralized virus is pinocytized by the host cell (16,53), antibody inhibits uncoating (30), antibody

inhibits the transfer of virus from intracytoplasmic vesicles (16), or antibody influences viral nucleic acid degradation (38).

Silverstein and Marcus (53) have reported that the majority of neutralized Newcastle disease virus elutes from HeLa cells. In constrast, neutralization of poliovirus suppresses elution from HeLa cells (37). Although pinocytosis of neutralized virus can occur as determined by electron microscopy, the majority of virions remain extracellular (14,15,16,53). It was concluded that antibody blocked pinocytosis but, degradation of neutralized virus immediately after entry into the cell was considered an alternative explanation.

Mandel (35) reported that if poliovirus were first neutralized and then adsorbed to HeLa cells, entry into the cell did not occur. However, if poliovirus were first adsorbed to the cell and then neutralized entry occurred (35, 38). Thus, entry of neutralized virus depends on whether virus is neutralized before or after adsorption to the cell.

Poliovirus adsorbs to HeLa cells between 0 and 5^{o} without entry into the cell as determined by sensitivity to neutralizing antibody. Once virus is within the cell, it is not sensitive to neutralization and can not be recovered by acid treatment of the cell. After 60 min. at 22 to 27^{o}, poliovirus was within the cell, resistant to antibody, and could not be recovered by acid treatment of the cell (35,39).

Infectious virus can be recovered after lysis of the cells with sodium dodecyl sulfate. Entry and uncoating of the virus is so rapid at 37° that infectious virus can not be recovered by lysis of the cell (39).

Poliovirus first adsorbed to cells at 2°, and then neutralized, can be dissociated from the cell by acid treatment with subsequent dissociation of antibody from virus. After 60 min. there was no decrease in the amount of infectious virus recovered at 2°, but there was a decrease at 37° (35,38). No intermediate temperatures were studied and no attempts were made to recover intracellular neutralized virus. Mandel (35,38) concluded that neutralized poliovirus enters the cell at 37° but not at 2°. In contrast, no entry occurs if poliovirus was neutralized before adsorption (35).

Several reports indicate that antibody may attach to nonspecific areas on the virus and consequently not neutralize the virus (3,29,51,57). However, anti-γ globulin antibody can react with the antibody attached to the virus and may neutralize the virus (3,29). Neutralization was reported to be the result of an increase in virus-antibody complex size (31). The large complexes were either blocked from adsorption or entry into the cell (31).

Although neutralized poliovirus is reactivated by papain or exposure to pH 4.0, antibody remains associated with the virus as determined by neutralization with anti-γ globulin serum (31). From these results, Keller (31) suggested that

neutralized virus is reactivated by modifying the configurational state of the antibody. Consequently, reactivated virus is infective even though antibody is still associated with the virus.

Events during and after virus entry

It has been generally assumed that ether-labile viruses such as vaccinia (1,13,14,49), Newcastle disease (46,53), parainfluenza SV5 (8), influenza (15), and herpes simplex (17,24,52) were pinocytized by the host cell as intact virions. Uncoating of ether-stable enteroviruses was reported to occur extracellularly at the cell membrane (9,22,23). Recent evidence refutes these hypotheses and indicates quite conclusively that entry of ether-labile viruses such as herpes simplex (43), influenza (44), and Sendai (45), begins with extracellular digestion of the viral coat and the host cell cytoplasmic membrane at the point of contact with subsequent entry of the viral nucleoprotein into the cytoplasm. Radiochemical (27,28) and electron microscopic studies (43,44,45) substantiate this conclusion.

Although the viral capsid of poliovirus is modified by contact with the host cell receptor, the ether-stable virus is pinocytized *in toto* into pinocytic vesicles from which the nucleoprotein or nucleic acid enters the cytoplasm (39). However, this process may accompany an uncoating at the membrane of the vesicle. According to Mandel (39), pinocytosis

of ether-stable poliovirus occurs at 21 to 37^{o} but uncoating occurs only at 32 to 37^{o}.

Electron microscopy of influenza virus indicated that antibody added after viral adsorption did not interfer with attachment but did prevent digestion of the viral envelope (44). Intact virus-antibody complexes were within phagocytic vesicles and thus, antibody did not prevent pinocytosis. Rabbitpox-antibody complexes enter cells and degradation occurs within cytoplasmic vesicles but the viral cores are not degraded (30). In contrast, vaccinia viral cores are degraded (16). Degradation of neutralized vaccinia virus was further substantiated by labeling the viral DNA with ^{3}H-thymidine and determining the grain count by autoradiography. After 4 hrs at 37^{o}, the decrease in grain count with nonneutralized and neutralized virus was 2% and 36%, respectively (16). Intracellular viral degradation of Newcastle disease virus occurs only when the virus is neutralized (53). Dales and Kajioka (16) suggested that digestion of neutralized virus within cytoplasmic vesicles results from the action of a group of lysosome hydrolases.

After 120 min. at 37^{o}, 10 to 15% of intact viral RNA was in HeLa cells with nonneutralized poliovirus whereas little or none was present if virus was neutralized (38). Even though there was the same amount of degraded RNA with nonneutralized or neutralized virus, Mandel (38) proposed that antibody influences polio viral RNA degradation because

intact RNA was not detected with neutralized virus. However, more nonneutralized virus was uncoated than neutralized virus.

INTERACTION OF NONNEUTRALIZED AND NEUTRALIZED AVIAN INFECTIOUS BRONCHITIS VIRUS WITH THE CHICKEN EMBRYO KIDNEY CELL

I. Entry into the Cell

INTRODUCTION

The role of neutralizing antibody in blocking viral infection depends on whether virus is neutralized before or after adsorption to the cell. In the former, it is generally accepted that antibody interferes with viral adsorption and entry into the cell. Adsorption may occur but the majority of neutralized viruses usually do not enter the cells (30,35, 43,50). However, even though a larger number of vaccinia-antibody complexes remained at the cell surface, some virus-antibody complexes were also engulfed by the host cells (16). In contrast, if antibody is added after poliovirus adsorption, it does not prevent the virus from entering cells at 37° (35,38). When heterotypic antibody is added after influenza viral adsorption, it blocks digestion of the viral envelope at the cytoplasmic membrane. Nevertheless, the virus-antibody complexes are pinocytized <u>in toto</u> by the host cell (44). However, the addition of antibody to Newcastle disease virus releases the virus from the cell (53).

The present report describes the entry of nonneutralized and neutralized avian infectious bronchitis virus (IBV) into chicken embryo kidney cells. The effect of temperature and antibody on the interaction of virus with the host cell was investigated. The second paper will describe studies with ^{32}P-labeled IBV and the effect of neutralizing antibody on the intracellular interaction of virus with the host cell.

METHODS

Virus. The 113th passage of the Beaudette strain of avian infectious bronchitis virus (IBV-42) was used because the plaque assay method with this strain was accurate and reproducible (11). Aggregates of virus were removed with a 200 mμ. filter (56).

Avian infectious bronchitis virus-42 is not inactivated at 37^{o} for 120 min. in phosphate buffered saline (PBS) containing 3% new born calf serum (3% nbcs). The virus is stabilized to thermal inactivation by anions (26) and is pH stable (54).

IgG neutralizing antibody. To study the interaction of neutralized IBV with the chicken embryo kidney cell (CEKC), a biologically homotypic antiserum with a high concentration of 7 S (IgG) neutralizing antibody was prepared. An antiserum relatively free of 19 S (IgM) antibody was necessary since IgM antibody would cause more steric hinderance. Eight-month-old Single Comb White Leghorn cockerels were inoculated intranasally with 0.2 ml., 1.6×10^{6} embryo infective $doses_{50}$ (EID_{50}), of the Massachusetts strain of avian infectious bronchitis virus (IBV-41). Six weeks later the chickens were reinoculated with 0.1 ml., 0.8×10^{6} (EID_{50}), of virus.

Three weeks after the second inoculation, the chickens were fasted for 24 hrs and then exsanguinated. The sera were pooled and incubated in a water bath at 56° for 30 min. and then stored at -20° until tested for IgM and IgG neutralizing antibody. Separation of IgM and IgG antibodies was by centrifugation through a linear sucrose gradient (10 to 37% sucrose in 0.15 M-NaCl) in the SW39L Spinco rotor. Prior to centrifugation, 0.1 ml. of whole serum, diluted 2-fold in 0.85% NaCl solution, was layered on the gradient. Human hemoglobin (4.2 S) was used as a sedimentation marker. Centrifugation was at 100,000g for 16 hrs. By means of a polystylic pump attached to a needle in the bottom of the tube, the entire gradient was slowly moved upward through a 1/8 inch hole in the center of a tightly fitted lucite block. Teflon tubing, 1/8 inch, was attached to the top of the lucite block with chromatronix cheminert fittings. The location of neutralizing antibody in the gradient fractions was determined by the plaque reduction method (10) and the location of human hemoglobin by absorbance at 412 mμ. Sedimentation coefficients were estimated by the method of Martin and Ames (40).

Cell culture. Primary chicken embryo kidney cell (CEKC) cultures were prepared from 17- to 18-day-old embryos (11) and were grown in 60 mm. Falcon plastic Petri dishes using medium 199 supplemented with BME vitamins, BME amino acids, 5% newborn calf serum, and with 100 units of penicillin,

100 μg. of streptomycin, and 50 units of mycostatin/ml.

Four Petri dish cell cultures were inoculated with 0.5 ml. of viral inoculum for each sample tested. For virus titration, adsorption was at 37° for 90 min. unless indicated otherwise. After adsorption, the inoculum was decanted and 4 ml. of supplemented medium 199 containing 0.9% Noble agar was added to each cell culture. The cells were incubated at 37° in 85% relative humidity and 8% CO_2 for 3 to 4 days and then 0.1% neutral red stain was added. After 30 min. at 37° and 1 hr at 4°, the plaques were counted and recorded as plaque forming units (p.f.u.) per milliliter of inoculum.

Standard incubation procedure for viral adsorption and neutralization. After adsorption of virus for 30 min. at 4° the CEKC were washed with ice-cold phosphate buffered saline (PBS) containing 3% new born calf serum (3% nbcs), pH 7.3, to remove unadsorbed virus. Anti-IBV chicken serum at a concentration that would neutralize approximately 90% of the adsorbed virus was added. After 30 min. at 4°, the cells were washed with ice-cold PBS (3% nbcs), pH 7.3, to remove unreacted and unadsorbed antibody. This stage of the procedure was considered as time zero for the various experiments. The cells were then incubated at 37 or 25° and 4° was the control. Adsorption and neutralization was at 4° because viruses do not enter cells at this temperature (18,56). The above incubations for viral adsorption and neutralization at 4° and for the interaction of nonneutralized and neutralized virus at

37, 25, and 4° are referred to as the standard incubation procedure.

Reactivation of neutralized virus. After the standard incubation procedure, neutralized virus was reactivated at 15 min. intervals by washing the cells for 10 sec. with glycine-HCl buffer (0.1 N), pH 2.0, followed immediately by two washings with PBS (3% nbcs), pH 7.3. As a control, non-neutralized virus was treated similarly. A neutralized virus control was washed 3 times with only PBS (3% nbcs), pH 7.3. All buffers were ice-cold.

Horse anti-chicken globulin serum. Horse anti-chicken globulin serum was purchased from the Roboy Surgical Instrument Co. Inc., Washington, D. C.

RESULTS

IgG neutralizing antibody

Anti-IBV chicken serum was devoid of IgM neutralizing antibody but had a high concentration of IgG neutralizing antibody as determined by centrifugation through a linear sucrose gradient (Fig. 1). No IgM globulins were detected by polyacrylamide gel electrophoresis.

Virus entry

After viral adsorption at 4°, groups of CEKC were washed with PBS and incubated at 4° (control) and at either 25 or 37°. At various intervals of time, the extracellular virus was neutralized by adding anti-IBV chicken serum and incubating at 4° for 30 min. After neutralization, the cells were washed with PBS and overlayed with agar medium.

Viral entry proceeded linearly at 37 and 25° but the virus remained extracellular at 4° and therefore was sensitive to antibody. After 45 min. at 37°, entry was complete and subsequently antibody had no effect. At 25°, approximately 75% of the virus entered the cells in 120 min. (Fig. 2).

The entry rate constants (k) calculated from the relationship $\ln(V_o/V_t)t$, where V_o equals the input virus

Figure 1. IgG neutralizing antibody in anti-IBV chicken serum. Neutralizing antibody (●) and human hemoglobin (O), 0.1 ml. respectivity, were applied to a 10 to 37% preformed sucrose gradient. After centrifugation at 100,000g for 16 hrs, 0.15 ml. samples were collected dropwise. Neutralizing antibody and human hemoglobin (4.2 S) were detected by plaque reduction and by absorbance at 412 mμ, respectively.

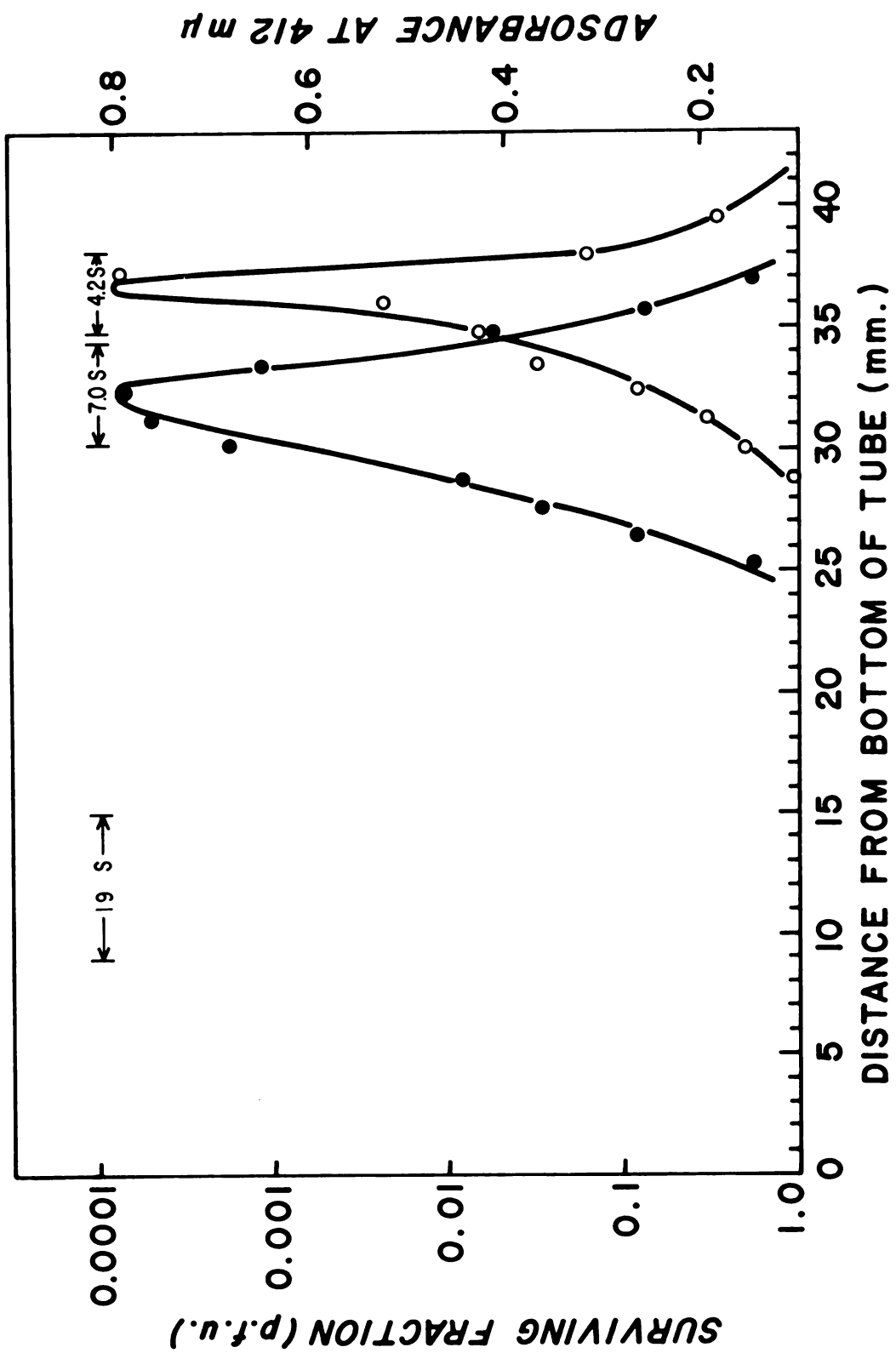
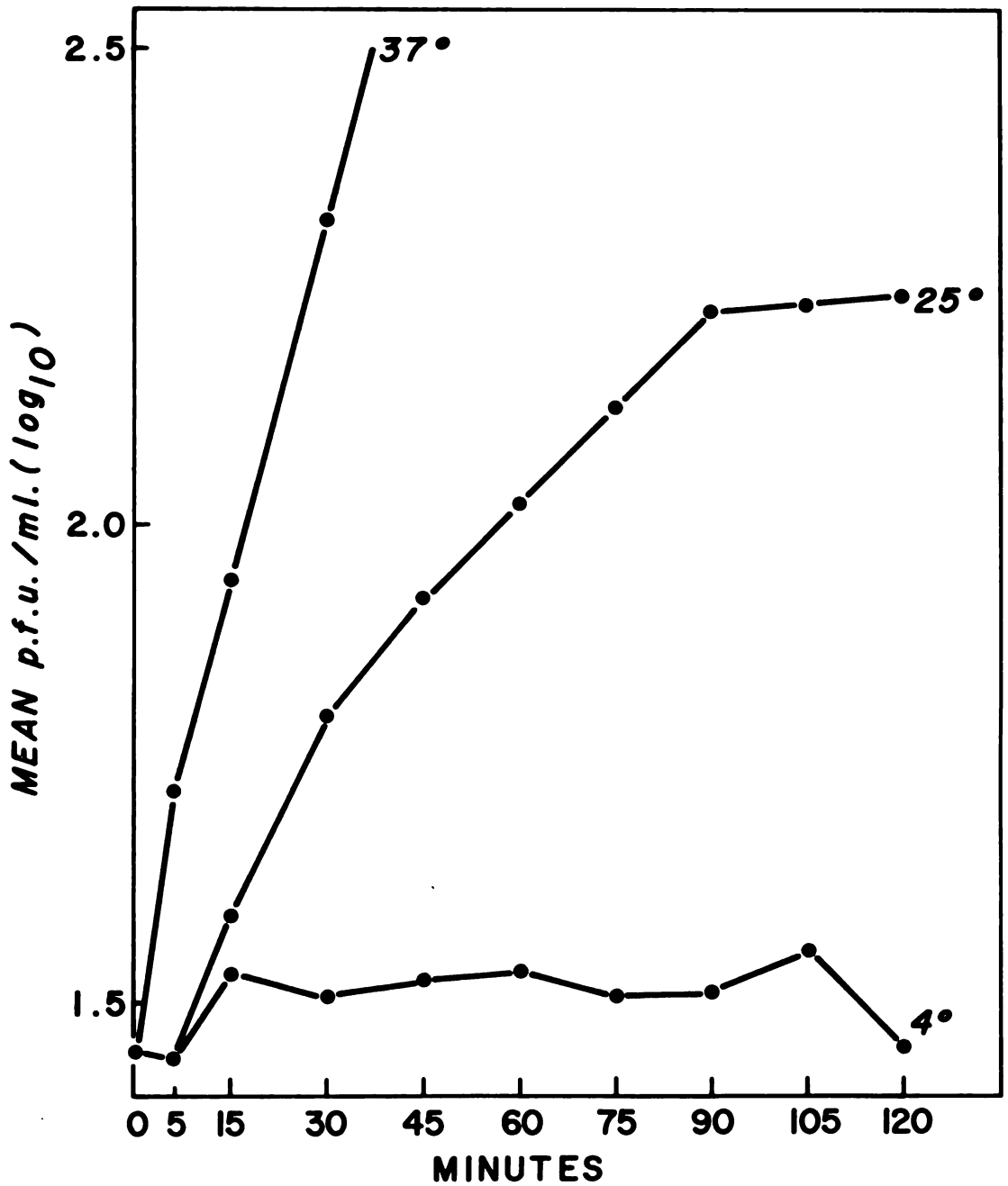

ADSORBANCE AT 412 mμ
0.8
0.6
0.4
0.2
4.2S
7.0S
19 S
SURVIVING FRACTION (p.f.u.)
0.0001
0.001
0.01
0.1
1.0
0
5
10
15
20
25
30
35
40
DISTANCE FROM BOTTOM OF TUBE (mm.)

Figure 2. Effect of temperature on the entry of IBV into CEKC as measured by the inability of antibody to neutralize intracellular virus.


2.5
2.0
1.5
MEAN p.f.u./ml. (log_{10})
37°
25°
4°
0 5 15 30 45 60 75 90 105 120
MINUTES

concentration and V_t equals extracellular virus at time t, were 15.9 min.$^{-1}$ and 4.5 min.$^{-1}$ at 37 and 25°, respectively. The same number of cells were used for each temperature. An Arrhenius plot of these data demonstrated a linear relationship between the logarithm of the reaction rate constant and the reciprocal of the absolute temperature of reaction. The energy of activation was calculated to be approximately 19 k. cal./mole.

Interaction of neutralized IBV with the CEKC

After the standard incubation procedure, the amount of neutralized virus that could be reactivated by acid treatment decreased linearly at 37 and 25° but the decrease was greater at 37° (Fig. 3A, B). After 120 min. there was no statistically significant (5% level of significance) reactivation at 37° but there was at 25°. In contrast, there was no decrease in reactivation at 4° (Fig. 3C). The mean virus titer after acid reactivation of neutralized virus decreased approximately 1 $\log_{10}$ within 120 min. at 37° (Fig. 3A). No neutralized virus was detected in the extracellular fluid. These results suggested that neutralized IBV merged with the cytoplasmic membrane at 25 and 37° but not at 4°. Brief acid treatment was without effect on the cells as determined by trypan blue. Likewise, there was no effect on the nonneutralized virus control. The virus was not inactivated by exposure to 37° or ice-cold glycine-HCl buffer, pH 2.0 (Fig. 3A, B, C). No increase in infectivity occurred when cells with neutralized

virus were washed 3 times with only PBS (3% nbcs), pH 7.3. However, a slight decrease in infectivity occurred which was probably due to further neutralization.

The per cent infectious virus recovered in the above experiment at hr intervals, as determined from the data of Figures 3 A, B, and C, are presented in Table 1.

The entry rate constants (k) for neutralized virus, calculated as previously described, were 0.12 min^{-1} and 0.09 min^{-1} at 37 and 25°, respectively. Entry of virus with antibody was also temperature-dependent but there was approximately a 100-fold decrease in the rate of entry as compared to virus without antibody. The energy of activation was calculated to be approximately 4 k. cal./mole.

Adsorption of IBV to CEKC and the effect of acid treatment on adsorbed virus

To determine the effect of acid treatment on IBV adsorbed to the cell, CEKC were inoculated with 1.5 X 10^2 p.f.u./ml. and incubated at 37°. At various intervals of time the cells were washed with PBS and then treated for 10 sec. with glycine-HCl buffer, pH 2.0, followed immediately by 2 washings with PBS (3% nbcs), pH 7.3. The controls were washed 3 times with only PBS (3% nbcs), pH 7.3. Cells were then overlayed with agar medium.

Attachment of IBV was so firm that brief acid treatment failed to dislodge the virus. Viral adsorption was rapid in that 50.41% of the viral inoculum adsorbed in 5 min.(Fig. 4).

Figure 3. Interaction of the IBV-antibody complex with CEKC at 37 (A), 25 (B), and 4° (C) as measured by acid reactivation of extracellular-neutralized IBV. After the standard procedures for viral adsorption, neutralization, and acid reactivation, cells with nonneutralized virus (△) or neutralized virus (●) were overlayed with agar medium. The neutralized virus control (O) was treated similarly but with PBS (3% nbcs), pH 7.3, substituted for the acid. Means and ranges of four determinations are indicated for each sampling interval.

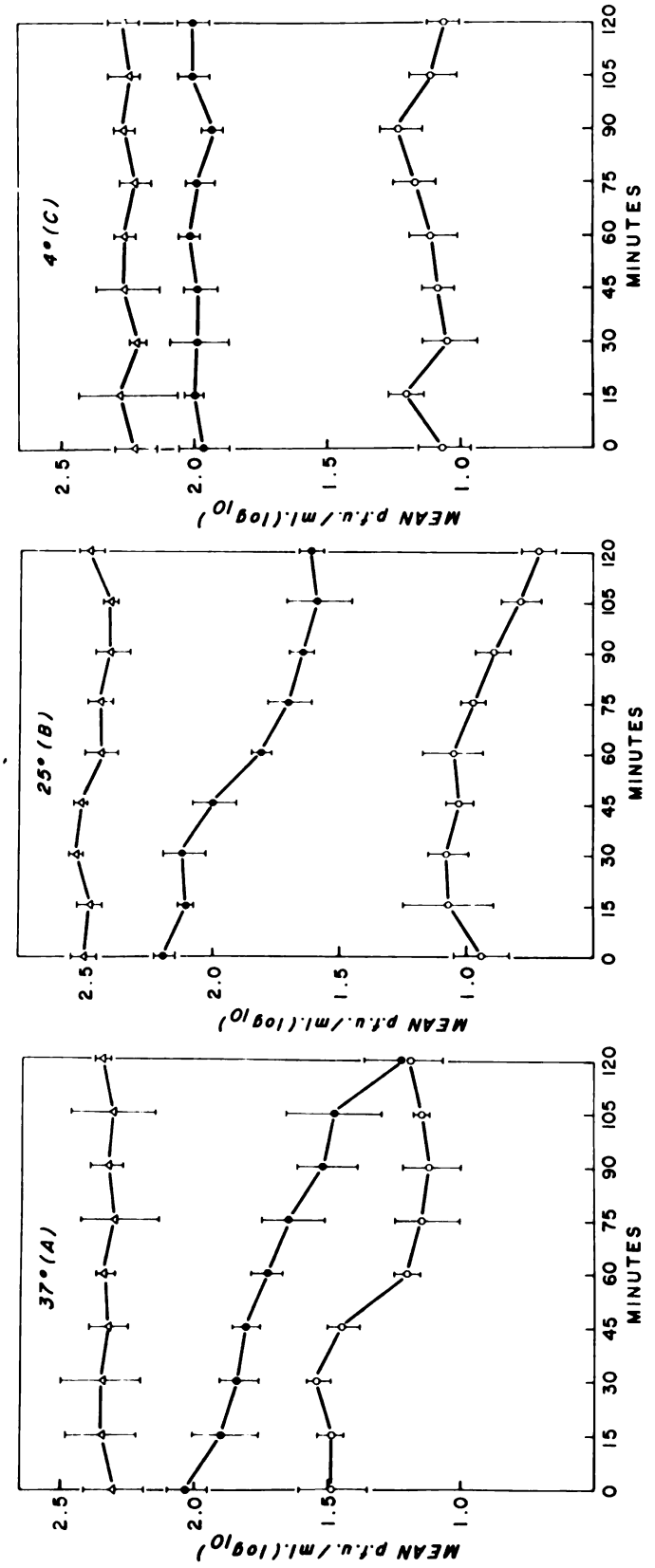

37° (A)
25° (B)
4° (C)
MEAN p.f.u./ml.(log10)
2.5
2.0
1.5
1.0
0
15
30
45
60
75
90
105
120
MINUTES

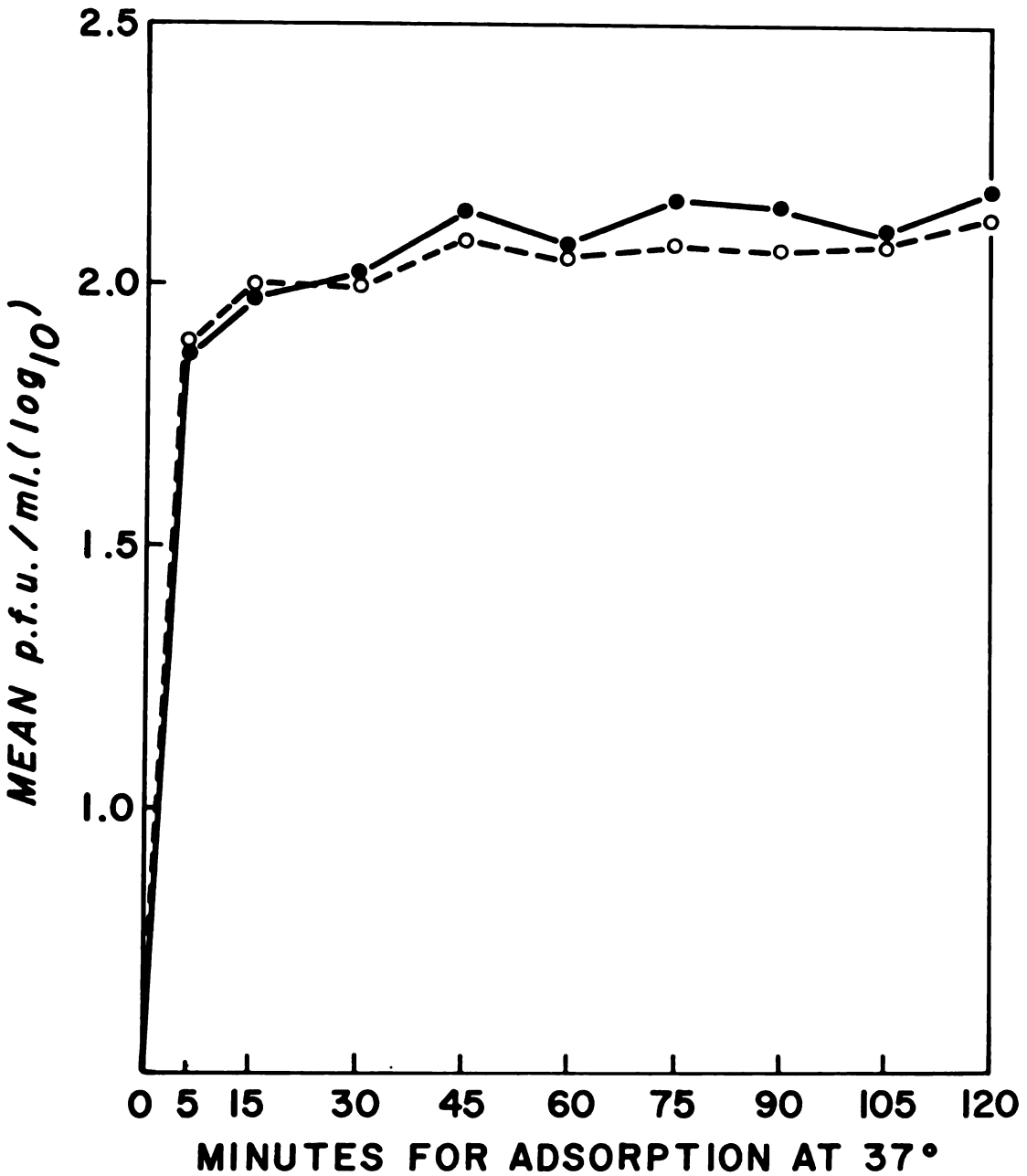
TABLE 1

Effect of temperature and time on the per cent infectious virus recovered by acid dissociation of antibody from IBV - cell complexes

	% Infectious virus recovered		
Temperature	0 min.*	60 min.	120 min.
37^0	54.3	24.7	8.4
25^0	48.3	22.8	13.6
4^0	55.3	58.8	56.0

*Immediately after the standard incubation procedure for viral adsorption and neutralization, the neutralized virus was reactivated by acid treatment. This point in the experiment was considered time zero.

Figure 4. Adsorption of IBV to CEKC and the effect of acid treatment on adsorbed virus. Cells were inoculated with IBV and incubated at 37°. At various intervals of time the cells were washed for 10 sec. with either glycine-HCl buffer (0.1 N), pH 2.0, (--O--) or PBS (3% nbcs), pH 7.3, (—●—) followed immediately by two washings with PBS (3% nbcs), pH 7.3.


2.5
2.0
1.5
1.0
MEAN p.f.u./ml.(log10)
0 5 15 30 45 60 75 90 105 120
MINUTES FOR ADSORPTION AT 37°

When the same experiment was done at 4^{o}, less adsorption occurred and approximately 6% of the virus dissociated from the cell.

Reactivation of neutralized IBV and the inability to reneutralize the virus with horse anti-chicken globulin antibody

Neutralized virus that was reactivated by papain or exposure to pH 4.0, still had antibody attached and subsequently, could be reneutralized with anti-γ globulin antibody (31).

To determine if chicken globulins were still associated with IBV after exposure of neutralized virus to pH 2.0, CEKC were inoculated with 1.2×10^{2} p.f.u./ml. Viral adsorption, neutralization, and acid reactivation were according to the standard procedures. Cultures were then divided into 3 groups to which was added PBS (3% nbcs), normal horse serum, and horse anti-chicken globulin antibody, respectively. After 30 min. at 4^{o}, the cultures were washed with PBS (3% nbcs), pH 7.3, and overlayed with agar medium.

The addition of horse anti-chicken globulin antibody to a mixture of virus and antiserum increases the amount of neutralization. Approximately 42% of the neutralized virus was reactivated by the acid treatment. Since the reactivated virus could not be reneutralized by horse anti-chicken globulin antibody, it was concluded that acid treatment at pH 2.0 dissociated antibody from the virus. Similar results were obtained with the controls, PBS (3% nbcs) and normal horse serum (Table 2).

TABLE 2

Reactivation of neutralized IBV and inability to reneutralize the virus with horse anti-chicken globulin antibody

Exp.*	Treatment **	% Neutralization	Treatment ***	% Reactivation	% Reneutralization
1	Neutralized virus + PBS (3%nbcs)	93.8	Reactivated virus + PBS (3%nbcs)	43.3	0
2	" "	87.3	" "	35.0	0
1	Neutralized virus + normal horse serum	88.8	Reactivated virus + normal horse serum	46.8	0
2	" "	86.1	" "	41.0	0
1	Neutralized virus + horse anti-chicken globulin antibody	95.8	Reactivated virus + horse anti-chicken globulin antibody	48.9	0
2	" "	93.4	" "	36.7	0

*Anti-IBV chicken serum was diluted 1:20 and 1:30 for experiments 1 and 2, respectively.
**The nonneutralized virus controls were not neutralized by the above treatments.
***The nonneutralized virus controls were not effected by the acid treatment.

DISCUSSION

Although viruses in general, including IBV reported in the present study, attach to cells at 4°, they do not enter the cells and remain vunerable to antibody (25,35,39, 44). However, IBV and other viruses (20,39) which enter cells at 25 to 37° are invulnerable to antibody.

It was possible to detect neutralized IBV at the cell surface by dissociating the antibody from the virus at pH 2.0 with accompanying reactivation of the virus. Acid treatment of chicken embryo kidney cells (CEKC) did not dissociate IBV from the cell, inactivate the viability of the cell, or interfere with the subsequent replication of IBV. The inability to detect chicken globulins attached to the virus after exposure to pH 2.0 indicated that antibody had dissociated from the virus. Even though neutralized poliovirus was reactivated by papain or exposure to pH 4.0, antibody was still associated with the virus (31).

Because reactivation of neutralized IBV decreased linearly with time at 37 and 25°, but not at 4°, it was proposed that neutralized IBV was either eluting from the cells or merging with the cytoplasmic membrane. However, it was not possible to detect significant elution of neutralized

virus. If neutralized virus merged with the cytoplasmic membrane, it could not be reactivated because the antibody would no longer be readily accessible to the acid. Therefore, it is proposed that neutralized IBV enters cells at 25 and 37° as does nonneutralized IBV.

Alternative hypotheses for the decrease in reactivation of neutralized virus are (i) a stable bond formed between antibody and virus or (ii) a large number of antibodies reacted with the virus and thus prevented complete reactivation by acid. These interpretations are not considered adequate because the majority of IBV, neutralized in vitro by high concentrations of antibody for a long incubation period, could be reactivated at pH 2.0 (54).

Entry of neutralized poliovirus into HeLa cells is reported to occur at 37° (35,38). In these experiments, the rate of entry into the cells at 37° occurred immediately and was exponential for 30 min. followed by a decrease in the rate of the reaction. In contrast, entry of neutralized IBV at 37° also occurred immediately but the rate of the reaction was slower and remained linear during the 120 min. of the experiments.

Entry of nonneutralized envelope viruses such as herpes simplex (43), influenza (44), and Sendai (45) involves a digestion of the viral envelope and the host cell cytoplasmic membrane at the point of contact. If heterotypic antibody is added after viral adsorption, it blocks digestion.

However, the virus-antibody complexes are pinocytized *in toto* by the host cell (44). Since the entry rate constant of nonneutralized IBV is at a greater magnitude than that for neutralized IBV, it is possible that the mechanism of entry is different for the two types of virions. Entry of IBV without antibody is probably by digestion of the viral envelope at the cytoplasmic membrane as is the case for other enveloped viruses (43,44,45). However, IBV with antibody may be pinocytized *in toto* by the host cell.

Studies with a virus that does not possess an envelope, poliovirus, have indicated that virus with antibody entered cells at the same rate and to the same degree as virus without antibody (38). In contrast, antibody effects the rate of entry of a virus containing an envelope such as IBV. With Newcastle disease virus, antibody prevents entry into the cell by influencing viral elution (53).

Homotypic antibody reacts only with the viral projections of IBV and does not react with the viral envelope (6). Therefore, antibody attached to IBV projections either reduced the rate of entry or influenced the mechanism of entry. Several reports have indicated that antibody may also attach to nonspecific areas of viruses and consequently, the virus can enter the cell and replicate (3,29,51,57). This report emphasizes that antibody effects the interaction of enveloped viruses with the host cell and supports the inference that viruses, neutralized after adsorption, will enter the host

cell. To delineate these phenomena, the interaction of neutralized IBV with the CEKC was studied by using ^{32}P-labeled virus as described in the following paper.

SUMMARY

The interaction of nonneutralized and neutralized avian infectious bronchitis virus (IBV) with the chicken embryo kidney cell (CEKC) was studied. Biologically homotypic antiserum containing only 7 S (IgG) antibody was used for neutralization. Entry of IBV into the cell, as measured by insensitivity of virus-cell complexes to antibody, occurred at 37 and 25° but not at 4°. To study entry of neutralized virus, IBV was adsorbed and neutralized at 4° and then the cells were incubated at either 4 (control), 25, or 37°. Extracellular neutralized IBV was detected by dissociating antibody from the virus at pH 2.0 for 10 sec. which subsequently reactivated the virus. This treatment did not dissociate the virus from the cell or injure the cell. Since it was not possible to detect any significant elution of neutralized virus, it was concluded that neutralized IBV merged with the cytoplasmic membrane at 37 and 25° but the neutralized virus remained extracellular at 4°. When the neutralized virus merged with the cytoplasmic membrane it was not accessible to the acid and consequently it could not be reactivated. The kinetics of entry of virus without antibody was at a faster rate than that for virus with antibody.

INTERACTION OF NONNEUTRALIZED AND NEUTRALIZED AVIAN INFECTIOUS BRONCHITIS VIRUS WITH THE CHICKEN EMBRYO KIDNEY CELL

II. Viral Degradation

INTRODUCTION

After avian infectious bronchitis virus (IBV) adsorbed to the chicken embryo kidney cells (CEKC) at 4^{o}, the subsequent addition of antibody neutralized the virus. Nevertheless, the virus with attached antibody entered the cell at 25 and 37^{o} but did not replicate (55). Studies with poliovirus indicated that virus with antibody also entered cells at 37^{o} (38). In contrast, viruses neutralized before inoculation generally do not enter cells (30,35,43,50).

The effect of antibody on the interaction of virus with the host cell is variable. Antibody may either stimulate viral elution from the cell (30,53) or suppress elution (37, 38). Intracellular virus with antibody attached is only partially uncoated (30) and the viral nucleic acid is not released (30,38) or is uncoated with release and subsequent degredation of the viral nucleic acid (16,38).

The present paper describes the effect of antibody on the interaction of ^{32}P-labeled IBV with the CEKC and the degradation of virus without and with antibody.

SCH
P.O
PHY

1-

2

1-
0

CH
AU

AD

c/

"a

nn

OC
NC
NC
Di
Ca
CO

Fo

ORDER NO. | DATE | ISSUE | W.O. NO. | YEAR

3 . 8

SCHOOL Mich S.U.

P.O. NO. 252135 Acct.#21-2541-07

PHYS. DESC. 1 book Bd.

No. Pos.

/ Total

LC C/R SCH.

4 1	Copyright	
4 2	Not C/R	R
4 3	Pos. to C/R	W
4 4	Pos. to School	B

1 2 25

Kn

	PUBL. NO.	NO.PAGES	PRICE 1 POS.	AUTHOR NAME (LAST, FIRST, MI.)	
1–2	25 31 / 16 22	33 36 / 32 35	37 = P 50 54	39	57 Format A / 77 Format B
2,1	6920940	70		STINSKI, Mark E.	

		Cust. No.			Adv. Pay No.	Adv. Pay Amt.
1–2	3–8	10 16	17 22		45 50	51 57
0 0			0 1 4 2 0 0			

CHG. AUTH.	1–2	9–11	QTY. 13 15	16 22	23 27	Service or Product	Advance Payments NO.	AMT.	FROM
	0 9	X		PD00421	00001	Micro and Abst. @ $20.00 Ea.			
	0 9	X		PD00421	00002	Micro and Abst. @ $25.00 Ea.			
	0 9	X		PD00421	00003	Publ. Abst. only @ $15.00 Ea.			
	0 9	X		PD00429	00000	Publish Diss. & Store Negative @ $12.00 Ea.			
	0 9	X		PD00422	00000	Copyright Fee Not Paid in Advance			
	0 9	X		PD00422	00001	Copyright Paid in Advance			
	0 9	X		PD00433	00000	Extra Pages in Abst. @ $2.40 per page.			
	0 9	X		PD00434	00000	Extra Listings in DA @ $2.00 Ea.			
	0 9								
	0 9	4 2 6		Format -B-		Positive Copies to C/R Office and/or School			
	0 9								

ADDRESS

T.P. nnbc.i
Abst. (2)
ii - vii
1-61

c/r p. ____ as rec'd
____ made by ed.
____ stripped by ed.

"a" pp. ____ by author
____ by ed.

nnbc ✓

OC ____ Lt. & dk.type ____
NOCbg ✓ Pap. prnt. ____
NOC ____ Mntd. illus. ____
Ditto ____ Glos. photo ____
Carbon ____ Color photo ____

CONDITION:
Summary, pg 56, shorter than D.A.

Foreign Text

METHODS

Plaque assay of avian infectious bronchitis virus (IBV) in chicken embryo kidney cells (CEKC), standard procedures for viral adsorption and neutralization at 4^{o}, and the preparation of IgG neutralizing antibody were described in the preceding paper (55).

^{32}P-labeling of IBV. Twenty-four hr old CEKC cultures in supplemented medium 199 were washed with phosphate-free Hanks' (PFH) solution at pH 7.0. Citrate, 0.001 M, was substituted for the phosphates in Hanks' balanced salt solution and the pH was adjusted with 1.4% $NaHCO_3$. To remove residue phosphates, the cells were incubated at 37^{o} for 24 hrs in phosphate-free medium 199.* During this period the phosphate-free medium was replaced twice.

The stock virus was dialyzed for 72 hrs against PFH and then 2 X 10^{7} p.f.u./ml. were added to the cells. After incubation at 37^{o} for 90 min., the inoculum was decanted. Phosphate-free medium supplemented with 5% newborn calf serum dialyzed for 72 hrs against PFH and with ^{32}P as carrier free orthophosphate, 0.02 mC./ml., was added to the cells.

---

*Grand Island Biological Co., Grand Island, New York, U.S.A.

Infected cultures were incubated approximately 72 hrs and then frozen and thawed 3 times in the medium. The mixture of medium and cell lysate was collected and is referred to as the "crude" virus preparation.

Purification of virus. The crude virus was centrifuged at 10,000g for 30 min. and then the supernatant fluid was collected and centrifuged at 78,000g for 2 hrs. After discarding the supernatant fluid, the sediment was washed, resuspended to original volume in 0.02 M phosphate buffer, pH 7.2, and subjected to a second cycle of low and high speed centrifugation. Sediments were washed and suspended to 1/10 the original volume in 0.02 M-phosphate buffer, pH 7.2. Two milliliters of virus were placed on a diethylaminoethyl (DEAE)-cellulose column (1 X 10 cm.) and the column was washed with 0.02 M-phosphate buffer until the radioactivity in the effluent was at background count. Virus was eluted from the column with 0.45 M-NaCl in 0.02 M-phosphate buffer, pH 7.2. The eluate was collected in successive 2 ml. amounts, dialyzed against PFH for 72 hrs, and tested for both infectivity (p.f.u.) and radioactivity (counts/min.). Fractions with 1×10^3 p.f.u./ml. or greater were pooled and when necessary concentrated by dialysis against polyethylene glycol until the radioactivity was 1×10^4 counts/min./ml. or greater. After concentration, the viral preparation was dialyzed against phosphate buffered saline solution free of magnesium and calcium, pH 7.5.

Protein was determined by the Folin phenol test (33).

Density gradient centrifugation after DEAE-cellulose chromatography was not necessary.

Radiochemical procedures. Radioactivity was measured in a Mark I Nuclear Chicago liquid scintillation counter using the solvent system described by Bray (7). To determine the acid-soluble radioactivity, crystalline bovine albumin and then trichloroacetic acid were added to the samples with final concentrations of 0.2% and 10%, respectively. The reaction mixtures were held at 4° for 2 hrs and then the precipitates were sedimented by centrifugation at 2,200*g* for 1 hr. A sample of the supernatant fluid, 0.5 ml., was removed and the precipitate was resuspended in the remaining supernatant fluid. The pH was adjusted to neutrality with 1 N-NaOH and radioactivity was measured. After determining the amount of radioactivity in the supernatant fluid and the precipitate, the per cent acid-soluble activity was calculated.

Samples labeled with ^{32}P were classified as whole virus (acid-insoluble and RNase resistant), intact RNA (acid-insoluble but RNase sensitive), or degraded viral material (acid-soluble). Equal volumes of sample and RNase, 20 μg./ml. in Tris buffer (0.1 M), pH 7.5, or sample and Tris buffer were mixed and incubated at 37° for 15 min. Acid-soluble radioactivity before RNase treatment was considered to be that of degraded viral material. After RNase treatment,

radioactivity that was acid-insoluble was considered to be that of whole virus and the increase in acid-soluble activity due to RNase was attributed to intact RNA.

RESULTS

Differential centrifugation and DEAE-cellulose chromatography for purification of ^{32}P-labeled IBV

Although 99% of the extraneous radioactivity was removed by centrifugation, 63% of the original infectious virus was recovered. The increase in specific activity, p.f.u./counts/min., and specific infectivity, p.f.u./mg. protein, indicated that nonviral material was being removed by differential centrifugation (Table 1). After DEAE-cellulose chromatography, there was a direct relationship between the concentration of virus, p.f.u./ml., and the amount of radioactivity, counts/min./ml. (Table 1). Even though there were differences in the viral concentrations, the specific activity in the respective fractions remained essentially the same.

Washing the DEAE column thoroughly before elution of the virus with 0.45 M-NaCl, removed some infectious virus and a large amount of viral aggregates remained on the column after elution. Consequently, the yield of infectious virus was low. Only 0.6% of the original radioactivity was recovered.

Protein in the respective fractions was not sufficient for accurate determination.

If a mixture of labeled non-infected CEKC medium, cell lysate, and non-labeled virus were subjected to the same purification procedure, there was little ^{32}P radioactivity associated with the virus.

Ethyl ether fractionization and sodium dodecyl sulfate solubilization of ^{32}P-labeled IBV

Equal volumes of purified virus and ethyl ether were mixed and incubated at 25° for 2 hrs in closed tubes that were shaken vigorously every 30 min. The mixture was centrifuged at 600g for 15 min. and the radioactivity of the ether layer, ether-water interface, and water layer was determined. Layers such as these with influenza virus have been reported to contain lipid, lipoprotein, and nonlipid, respectively (28). Radioactivity of the nonlipid was considered to be associated mainly with the viral RNA.

A small amount of the ^{32}P was in the lipid fraction but greater amounts were in the lipoprotein and nonlipid fractions (Table 2A).

To substantiate the amount of ^{32}P associated with the viral lipid and lipoprotein, purified virus was treated with 0.4% sodium dodecyl sulfate (SDS) at 25° for 15 min. The control was virus in phosphate buffered saline without magnesium or calcium.

Infectious virus was completely inactivated by 0.4% SDS and approximately 50% of the ^{32}P was soluble in trichloroacetic acid (Table 2B). Approximately the same amount of ^{32}P

TABLE 1

Differential centrifugation and DEAE-cellulose chromatography for purification of ^{32}P-labeled IBV

Sample	Infectivity (p. f. u. /ml.)	Radioactivity (counts/min. /ml.)	Specific Activity (p. f. u. /counts/min.)	Specific Infectivity (p. f. u. /mg. protein)
Crude	2.30×10^6	7.95×10^6	0.29	2.88×10^5
10, 000 g supernatant fluid	5.00×10^6	3.36×10^7	0.15	9.09×10^5
78, 000 g sediment, 1st cycle	4.40×10^6	7.84×10^5	5.61	1.34×10^7
78, 000 g sediment, 2nd cycle, concentrated 10-fold	3.14×10^6	3.10×10^6	10.13	8.72×10^7
DEAE, 0.45 M-NaCl effluent number:				
1-5	not detectable	*	**	not detectable
6	400	670	0.60	1-20
7	4, 700	9, 400	0.50	
8	4, 300	6, 350	0.68	
9	4, 060	7, 800	0.52	
10	2, 600	5, 290	0.49	
11	2, 500	4, 460	0.56	
12	1, 500	3, 320	0.45	
13	1, 140	2, 220	0.51	
14	700	1, 630	0.43	
15	800	1, 590	0.50	
16	727	1, 460	0.50	
17	400	825	0.49	
18	600	1, 190	0.50	
19	370	895	0.41	
20	267	514	0.52	

*Normal background counts

**The mean specific activity of the DEAE fractions was 0.51 ± 0.13

TABLE 2

Ethyl ether fractionization (A) and sodium dodecyl sulfate solubilization (B) of ^{32}P-labeled IBV.

A. Ethyl Ether Fractionization

Fraction	% ^{32}P
Lipid	5.0 $\pm$ 2.1
Lipoprotein	42.6 $\pm$ 4.3
Nonlipid	52.6 $\pm$ 2.1

B. SDS Solubilization

Treatment of Virus	p. f. u. /ml.	% ^{32}P trichloroacetic acid-soluble
PBS at 25^{0} for 15 min.	1.1 X 10^{3}	2.9 $\pm$ 0.2
0.4 %SDS in PBS at 25^{0} for 15 min.	0	50.0 $\pm$ 2.3

in the viral lipid and lipoprotein was detected by both ethyl ether fractionization and treatment with SDS.

Adsorption of ^{32}P-labeled IBV to CEKC

After incubating one group of cells at 4^{o} and another group at 37^{o} for 30 min., the cells were inoculated with 2×10^{4} p.f.u./ml. of ^{32}P-labeled IBV. At certain time intervals, the amount of unadsorbed virus at 4^{o} and at 37^{o} was determined by infectivity and radioactivity (21,32,37).

Adsorption was exponential and approximately 20 and 30% of the inoculum adsorbed in 5 min. at 4 and 37^{o}, respectively (Fig. 1). Lesser adsorption occurred at 4^{o}. At the respective temperatures, the kinetics of adsorption when measured by infectivity or radioactivity were identical (Fig. 1).

Effect of temperature on the distribution of ^{32}P in CEKC with nonneutralized and neutralized ^{32}P-labeled IBV

Using 1×10^{4} p.f.u./ml. of ^{32}P-labeled IBV, viral adsorption and neutralization were according to the standard incubation procedure. Cells with nonneutralized and neutralized ^{32}P-labeled IBV were washed 3 times with phosphate buffered saline (PBS), pH 7.5, to remove unadsorbed virus and then 1 ml. of PBS was added. Groups of CEKC cultures were incubated at 4^{o} (control) and at either 25 or 37^{o}. At 15 min. intervals, the extracellular fluid was removed and the cells were suspended in 0.5 ml. of PBS. The ^{32}P radioactivity associated with the cells and in the extracellular fluid was determined.

At 37° for 75 min., the ^{32}P cell-associated activity decreased linearly and simultaneously the activity in the extracellular fluid increased (Fig. 2A). In contrast, the ^{32}P cell-associated activity decreased slightly at 25° and in the 4° control, there was no change in the ^{32}P cell-associated activity. At all temperatures, there was no difference in the interaction of nonneutralized and neutralized IBV with the host cell (Fig. 2A, B, C).

The extracellular fluids were tested for elution of infectious virus, ^{32}P, and degraded viral material. At 37°, approximately 1% of the adsorbed infectious virus eluted from the cells with nonneutralized virus (Table 3). It is possible that some of the adsorbed infectious virus was no longer infectious after elution. The majority of the radioactivity eluted was probably due to acid-insoluble virus and viral lipoprotein components. Approximately, $\frac{1}{4}$ of the activity was due to degraded viral material. At the 5% level of significance there were no significant differences ($P > 0.10$) between nonneutralized or neutralized IBV with respect to the per cent ^{32}P eluted or the per cent degraded viral material in the extracellular fluid (Table 3). Antibody did not enhance elution of virus. Viruses with antibody were apparently degraded at the same rate as viruses without antibody.

At 25° for 120 min., the small amount of ^{32}P activity in the extracellular fluid was mainly acid-insoluble.

Figure 1. Adsorption of ^{32}P-labeled IBV to CEKC. The per cent unadsorbed virus at 4 (———) and 37^{o} (-----) was determined by ^{32}P (O,Δ) and infectivity (●,▲).

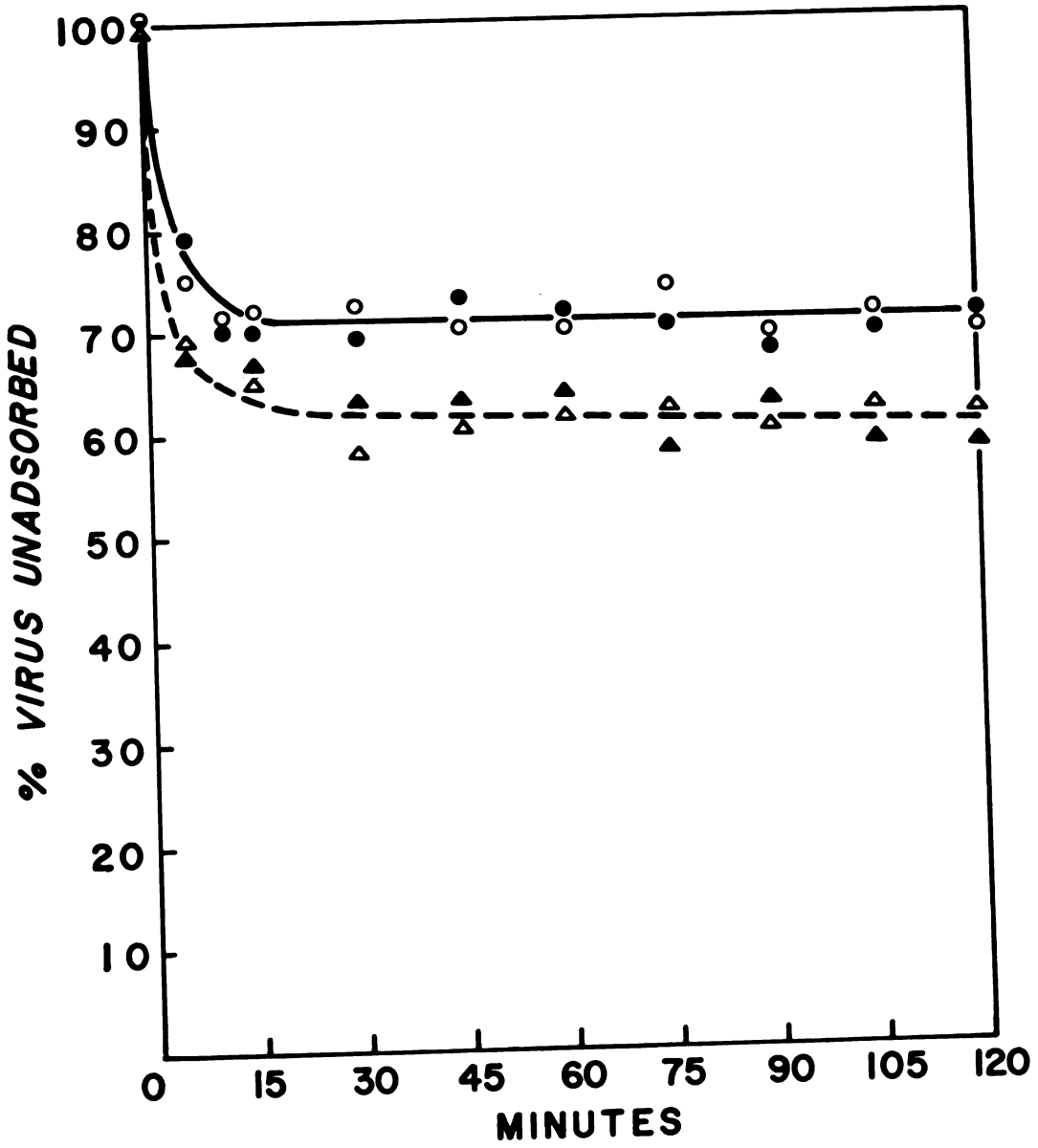

100
90
80
70
60
50
40
30
20
10
% VIRUS UNADSORBED
0
15
30
45
60
75
90
105
120
MINUTES

Figure 2. Effect of temperature on the distribution of ^{32}P in the extracellular fluid (-----) and that associated with the cell (———) for CEKC with nonneutralized (▲,△) or neutralized (●,○) ^{32}P-labeled IBV. After the standard incubation procedure for viral adsorption and neutralization, groups of CEKC were incubated at 37 (A), 25 (B), and 4° (C), respectively.

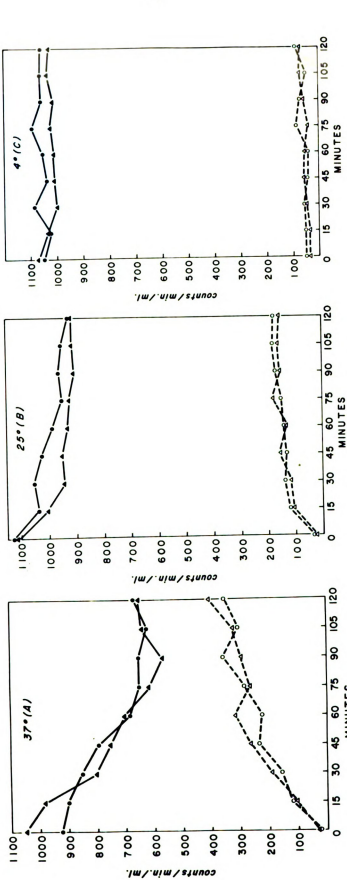

37° (A)
25° (B)
4° (C)
counts / min. / ml.
MINUTES
0
15
30
45
60
75
90
105
120
100
200
300
400
500
600
700
800
900
1000
1100

TABLE 3

Elution of infectious virus, ^{32}P, and degraded viral material from CEKC with ^{32}P-labeled IBV, nonneutralized and neutralized

Minutes at 37°	% Infectious Virus Eluted	% ^{32}P Eluted from CEKC		% ^{32}P Trichloroacetic acid-soluble	
		Non-neutralized Virus*1	Neutralized Virus*1	Non-neutralized Virus*2	Neutralized Virus*2
0	0.7	1.8	2.8	3.1	2.9
15	0.7	11.5	13.4	14.6	17.9
30	0.8	23.8	18.3	15.2	18.3
45	0.9	34.9	29.9	19-	19.5
60	0.9	44.9	32.6	16.2	19.6
75	0.9	43.0	42.6	23.9	24.3
90	1.0	52.5	54.5	24.2	23.9
105	1.1	50.6	49.9	24.1	23.9
120	1.1	61.6	52.6	24.2	24.0

*1 There was no significant difference ($p > 0.10$) in the per cent ^{32}P eluted from CEKC with nonneutralized or neutralized virus.

*2 There was no significant difference ($p > 0.10$) in the per cent ^{32}P trichloroacetic acid-soluble activity in the extracellular fluid of CEKC with nonneutralized or neutralized virus.

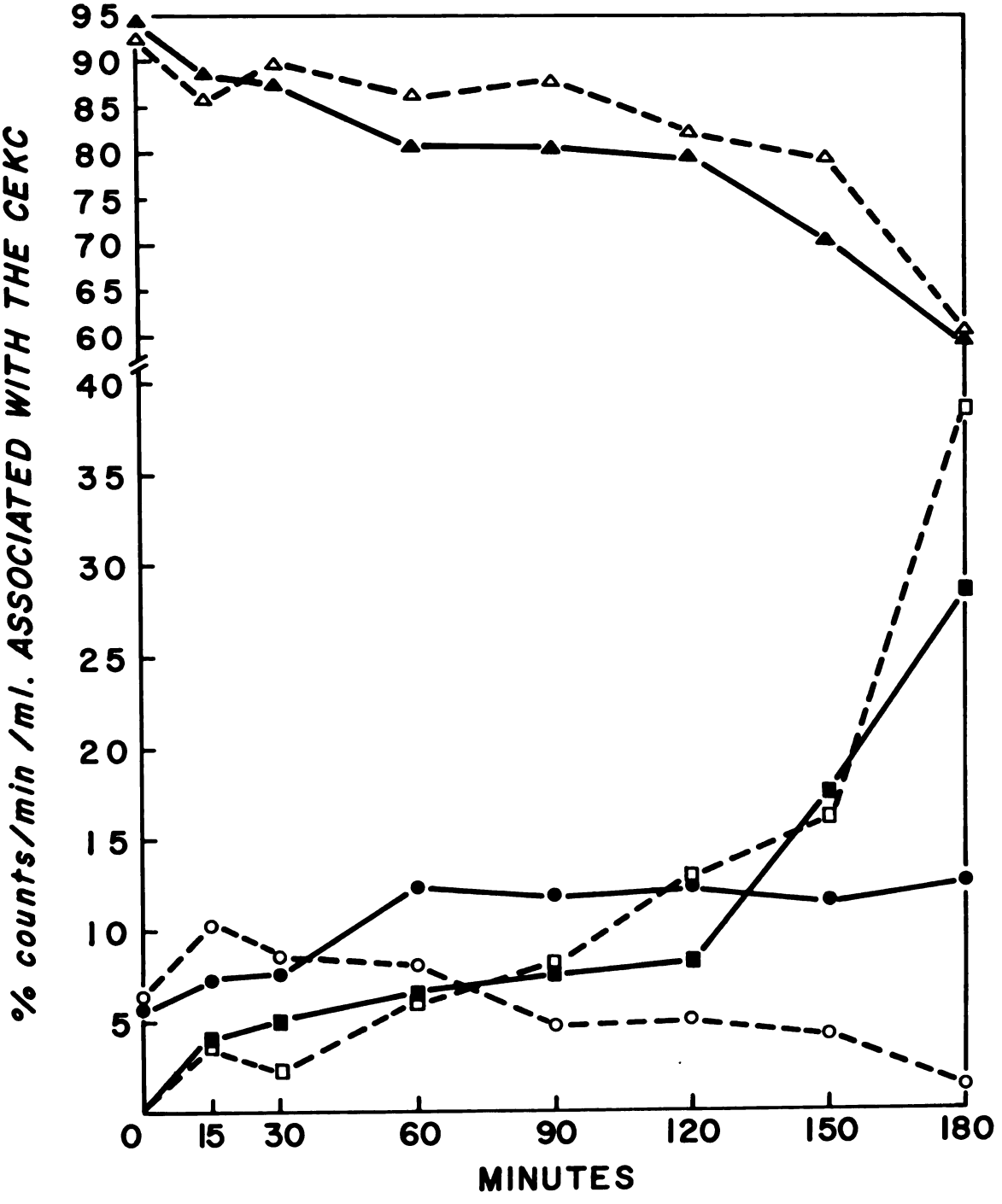
Degradation of nonneutralized and neutralized ^{32}P-labeled IBV in CEKC

Using 1 X 10^4 p.f.u./ml. of ^{32}P-labeled IBV, viral adsorption and neutralization were according to the standard incubation procedure and the cells were washed as previously described. The cells were incubated at either 4° (control) or 37°. At certain time intervals, the extracellular fluid was removed and the cells were washed with PBS, suspended in 0.5 ml. of double glass-distilled water, and lyzed by 4 cycles of freezing and thawing. The cells were then analyzed for acid-insoluble RNase resistant, acid-insoluble RNase sensitive, and acid-soluble material as previously described.

Freezing and thawing did not damage the virus. There was no increase in the ^{32}P acid-soluble activity in the 4° controls. However, approximately 3% of the activity was solubilized by 37° for 15 min. and subsequently there was a 5-6% increase in RNase-sensitive material. Therefore, some of the viral particles were probably damaged at 37°. No cell-associated degraded viral material was detected at time zero. The RNase-sensitive material at this time was probably due to externally adsorbed-virus damaged at 37° (Fig. 3).

At 37°, the same amount of whole virus and degraded viral material was associated with cells containing non-neutralized or neutralized virus. Maximum intracellular degradation of virus without and with antibody did not occur until after 120 min. After 60 min. at 37°, the amount of acid-insoluble, RNase sensitive material remained at

Figure 3. Degradation of ^{32}P-labeled IBV, nonneutralized (———) and neutralized (-----) in CEKC at 37°. The cells were analyzed for acid-insoluble, RNase resistant (▲,△), acid-insoluble, RNase sensitive (●,○), and acid-soluble material (■,□).


% counts/min /ml. ASSOCIATED WITH THE CEKC
95
90
85
80
75
70
65
60
40
35
30
25
20
15
10
5
0
15
30
60
90
120
150
180
MINUTES

approximately 12% in cells with nonneutralized virus but decreased to 1% in cells with neutralized virus (Fig. 3). This difference in the amount of acid-insoluble RNase sensitive material was most apparent at a time when the majority of neutralized virus was intracellular.

At 25° only a small amount, 8%, of degraded viral material was detected and there were no differences between nonneutralized and neutralized virus.

DISCUSSION

Studies on the interaction of neutralized ^{32}P-labeled avian infectious bronchitis virus (IBV) with the chicken embryo kidney cell (CEKC) established certain similarities as well as dissimilarities with other viruses (16,30,37,38, 53). Antibody had no effect on elution of IBV from CEKC. In contrast, antibody initiates elution of Newcastle disease virus from HeLa cells (53). However, with poliovirus the opposite occurs in that there is less elution of virus with antibody than without antibody (37,38).

It was previously proposed (55) that neutralized IBV enters CEKC by merging with the cytoplasmic membrane at 37 and 25°. The present results confirm that neutralized IBV enters CEKC at 37° and is subsequently degraded. Similar results have been reported with neutralized viruses of rabbitpox (30), vaccinia (16), and polio (38). At 25° IBV remains cell-associated and very little degradation occurs. It is possible that at 25° additional time might be required for degradation or the viral lipoprotein is actually removed from the virus but remains associated with the cytoplasmic membrane and is consequently acid-insoluble. At 25° poliovirus is not uncoated (39).

Even though it was established that IBV with antibody enters at a slower rate than IBV without antibody (55), there were no significant differences in the rate of degradation at 37°. Approximately the same per cent extracellular or intracellular degraded IBV material was detected. However, there was a difference in the amount of RNase sensitive material. In cells with neutralized virus, a decrease in RNase sensitive material occurred and consequently, little or none could be detected after 180 min. A decrease in the amount of intact RNA from neutralized ^{32}P-labeled poliovirus occurs within 15 min. at 37° (38). However, experiments with ^{3}H-labeled vaccinia DNA indicated that the viral DNA from neutralized virus is degraded within 1 to 2 hrs at 37° (16).

Studies with ^{32}P-labeled poliovirus (38) suggested that antibody may either suppress a reaction necessary for the release of intact viral RNA or stimulate a reaction which rapidly degrades the released RNA. Of the two hypotheses, the latter was considered to be a more favorable explanation of the results. The results obtained with neutralized IBV confirm that degradation occurs but further studies are necessary to differentiate between the degradation of virus without and with antibody.

It is possible that the nucleoprotein of nonneutralized IBV escapes from the virus at the cytoplasmic membrane as does that of viruses such as herpes simplex (43), influenza (44), and Sendai (45). With antibody attached, IBV may be

pinocytized by the host cell and completely degraded within pinocytic vesicles by lysosomal enzymes. The RNase in pinocytic vesicles could destroy the viral RNA and consequently prevent replication. According to Morgan and Rose (44), heterotypic antibody prevented digestion of influenza viral envelope at the cytoplasmic membrane but the virus-antibody complexes appeared to enter the cell by pinocytosis. However, the accumulation of degraded viral material in the extracellular fluid suggested that homotypic antibody did not prevent digestion of the IBV envelope. Neutralized vaccinia virus was pinocytized and then degraded within cytoplasmic vesicles (16).

According to Berry and Almeida (6), biologically homotypic antibody reacts with the viral projections but not with the envelope of IBV. In the present investigation homotypic antibody was also used. Neutralized IBV entered the CEKC but no replication occurred. Therefore, it is hypothesized that the attachment of antibody to the viral projections of IBV either inhibits the uncoating of the viral nucleoprotein or influences the degradation of the viral RNA. The latter hypothesis is more favorable.

SUMMARY

There was an identical relationship between adsorption of infectious virus and radioactivity of ^{32}P-labeled avian infectious bronchitis virus (IBV) purified by centrifugation and chromatography. Antibody did not enhance or suppress elution of IBV from chicken embryo kidney cells (CEKC). The distribution of ^{32}P from nonneutralized and neutralized labeled IBV in CEKC changed rapidly at 37^{o} but not at 25 or 4^{o}. At 37^{o}, virus without antibody was degraded at the same rate as virus with antibody. Even though virus without and with antibody entered cells at 25^{o}, there was very little detectable degradation. After 1 hr at 37^{o}, the per cent intracellular RNase sensitive material in cells with nonneutralized virus remained essentially the same but a decrease occurred in cells with neutralized virus. These data support the inference that neutralized virus is degraded intracellularly and suggest that the degradation of the virus is influenced by the attachment of antibody to the viral projections and consequently viral replication is prevented.

LITERATURE CITED

1. Adams, W. R., and A. M. Prince. 1957. An electron microscopic study of incomplete virus formation. Infection of Ehrlich ascites tumor cells with "chick embryo-adapted" Newcastle disease virus (NDV). J. Exptl. Med. 106:617-626.

2. Akers, T. G., and C. H. Cunningham. 1968. Replication and cytopathogenicity of avian infectious bronchitis virus in chicken embryo kidney cells. Arch. Ges. Virusforsch. 25:30-37.

3. Ashe, W. K., and A. L. Notkins. 1967. Kinetics of sensitization of herpes simplex virus and its relationship to the reduction in the neutralization rate constant. Virology 33:613-617.

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. Berry, D. M. 1967. Intracellular development of infectious bronchitis virus. Nature 216:393-394.

6. Berry, D. M., and J. D. Almeida. 1968. The morphological and biological effects of various antisera on avian infectious bronchitis virus. J. gen. Virol. 3:97-102.

7. Bray, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. 1:279-285.

8. Compans, R. W., K. V. Holmes, S. Dales, and P. W. Choppin. 1966. An electron microscopic study of moderate and virulent virus-cell interactions of the parainfluenza virus SV5. Virology 30:411-426.

9. Crowell, R. L. 1966. Specific cell-surface alteration by enteroviruses as reflected by viral attachment interference. J. Bacteriol. 91:198-204.

10. Cunningham, C. H. 1963. A laboratory guide in virology. 6th 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. Dales, S., and L. Siminovitch. 1961. The development of vaccinia virus in Earle's L strain cells as examined by electron microscopy. J. Biophys. Biochem. Cytol. 10:475-503.

14. Dales, S. 1962. An electron microscope study of the early association between two mammalian viruses and their hosts. J. Cell Biol. 13:303-322.

15. Dales, S., and P. W. Choppin. 1962. Attachment and penetration of influenza virus. Virology 18:489-493.

16. Dales, S., and R. Kajioka. 1964. The cycle of multiplication of vaccinia virus in Earle's strain L cells. I. Uptake and penetration. Virology 24:278-294.

17. Epstein, M. A., K. Hummeler, and A. Berkaloff. 1964. The entry and distribution of herpes virus and colloidal gold in HeLa cells after contact in suspension. J. Exptl. Med. 119:291-302.

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

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

20. Hahon, N., and K. O. Cooke. 1967. Primary virus-cell interactions in the immunofluorescence assay of Venezuelan equine encephalomyelitis virus. J. of Virol. 1:317-326.

21. Henry, C., and J. S. Youngner. 1963. Studies on the structure and replication of the nucleic acid of poliovirus. Virology 21:162-173.

22. Holland, J. J. 1962. Irreversible eclipse of poliovirus by HeLa cells. Virology 16:163-176.

23. Holland, J. J., and Hoyer, B. H. 1962. Early stages of enterovirus infection. Cold Spring Harbor Symp. Quant. Biol. 27:101-111.

24. Holmes, I. H., and D. H. Watson. 1963. An electron microscope study of the attachment and penetration of herpes virus in BHK21 cells. Virology 21:112-123.

25. Homma, M., and A. F. Graham. 1965. Intracellular fate of Mengo virus ribonucleic acid. J. Bacteriol. 89:64-73.

26. Hopkins, S. R. 1967. Thermal stability of infectious bronchitis virus in the presence of salt solutions. Avian Dis. 10:261-267.

27. Hoyle, L., and N. B. Finter. 1957. The use of influenza virus labelled with radiosulphur in studies of early stages of the interaction of virus with the host cell. J. Hyg. 55:290-297.

28. Hoyle, L., R. W. Horne, and A. P. Waterson. 1962. The structure and composition of the Myxoviruses. III. The interaction of influenza virus particles with cytoplasmic particles derived from normal chorio-allantoic membrane cells. Virology 17:533-542.

29. Iwasaki, T., and R. Ogura. 1968. Studies on neutralization of Japanese encephalitis virus (JEV). I. Further neutralization of the resistant virus fraction by an interaction between antivirus IgG antibody and IgG heterotype or allotype antibody. Virology 34:141-148.

30. Joklik, W. K. 1964. The intracellular fate of rabbitpox virus rendered noninfectious by various reagents. Virology 22:620-633.

31. Keller, R. 1968. Studies on the mechanism of the enzymatic reactivation of antibody-neutralized poliovirus. J. Immunol. 100:1071-1079.

32. Lawrence, W. C., and H. S. Ginsberg. 1967. Intracellular uncoating of type 5 adenovirus deoxyribonucleic acid. J. Virology 1:851-867.

33. Lowry, O. H., N. H. Rosenbrough, A. L. Farr, and R. L. Randal. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem., 193:265-275.

34. Lukert, D. P. 1967. Characterization of receptors and lysosomes of cells susceptible to infectious bronchitis virus. Ph. D. Thesis, Dept. of Veterinary Microbiology and Preventive Medicine, Iowa State Univ., Ames, Iowa.

35. Mandel, B. 1962. Early stages of virus-cell interaction as studied by using antibody. Cold Spring Hqrbor Symp. Quant. Biol. 27:123-136.

36. Mandel, B. 1962. The use of sodium dodecyl sulfate in studies on the interaction of poliovirus and HeLa cells. Virology 17:288-294.

37. Mandel, B. 1967. The interaction of neutralized poliovirus with HeLa cells. I. Adsorption. Virology 31:238-247.

38. Mandel, B. 1967. The interaction of neutralized poliovirus with HeLa cells. II. Elution, penetration, uncoating. Virology 31:248-259.

39. Mandel, B. 1967. The relationship between penetration and uncoating of poliovirus in HeLa cells. Virology 31:702-712.

40. Martin, R. G., and B. N. Ames. 1961. A method for determining the sedimentation behavior of enzymes: Application to protein mixtures. J. Biol. Chem. 236: 1372-1379.

41. McIntosh, K., W. B. Becker, and R. M. Chanock. 1967. Growth in suckling mouse brain of IBV-like viruses from patients with upper respiratory tract disease. Proc. Natl. Acad. Sci. 58:2268-2273.

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

43. Morgan, C., H. M. Rose, and B. Mednix. 1968. Electron microscopy of herpes simplex virus. I. Entry. J. Virol. 2:507-516.

44. Morgan, C. and H. M. Rose. 1968. Structure and development of viruses as observed in the electron microscope. VIII. Entry of influenza virus. J. Virol. 2:925-936.

45. Morgan, C., and C. Howe. 1968. Structure and development of viruses as observed in the electron microscope. IX. Entry of parainfluenza I (Sendai) virus. J. Virol. 2:1122-1132.

46. Mussgay, M., and J. Weibel. 1962. Early stages of infection with Newcastle disease virus as revealed by electron microscopy. Virology 16:506-508.

47. Nazerian, K., and C. H. Cunningham. 1967. Electron microscopy of the hemagglutinin of infectious bronchitis virus. Proc. 25th Ann. Meet. Electron Microscopy Soc. of America. 94-95.

48. Nazerian, K., and C. H. Cunningham. 1968. Morphogenesis of avian infectious bronchitis virus in chicken embryo fibroblasts. J. gen. Virol. 3:469-470.

49. Nielsen, G., and D. Peters. 1962. Elektronenmikroskopische Untersuchungen über die Initialstadien der Vaccine-Virusinfektion von HeLa-Zellen. Arch. Ges. Virusforsch. 12:496-513.

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

51. Seto, J. T., and Rott, R. 1966. Functional significance of sialidase during influenza virus multiplication. Virology 30:731-737.

52. Siegert, R., and D. Falke. 1966. Elektronenmikroskopische Untersuchungen über die Entwicklung des Herpesvirus hominis in Kulterzellen. Arch. Ges. Virusforsch. 19:230-249.

53. Silverstein, S. C., and F. I. Marcus. 1964. Early stages of Newcastle disease virus-HeLa cell interaction: An electron microscope study. Virology 23:370-380.

54. Stinski, M. F., and C. H. Cunningham. 1969. Neutralizing antibody complex of infectious bronchitis virus. J. Immunol. 102: (In press).

55. Stinski, M. F., and C. H. Cunningham. Interaction of non-neutralized and neutralized avian infectious bronchitis virus with the chicken embryo kidney cell. I. Entry into the cell. (Manuscript being prepared for publication.)

56. Wallis, C., and J. L. Melnick. 1967. Virus aggregation as the cause of the nonneutralizable persistent fraction. J. Virol. 1:478-488.

57. Webster, R. G., and W. G. Laver. 1967. Preparations and properties of antibody directed specifically against the neuraminidase of influenza virus. J. Immunol. 99:49-55.