CULTIVATION OF INFECTIOUS BRONCHITIS VIRUS IN CHICKEN EMBRYO KIDNEY CELLS

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY

Martha P. Spring

1960

CULTIVATION OF INFECTIOUS BRONCHITIS VIRUS IN CHICKEN EMBRYO KIDNEY CELLS

By

MARTHA P. SPRING

A THESIS

Submitted to the College of Science and Arts of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department of Microbiology and Public Health

1960

G-12179
11-7-60

ACKNOWLEDGMENTS

I wish to extend my appreciation to Dr. Charles H. Cunningham, Professor of Microbiology and Public Health, for his consideration, encouragement and guidance throughout the investigation and the preparation of this manuscript.

My appreciation is also extended to Dr. H. J. Stafseth, Professor (Emeritus), of Microbiology and Public Health, for his encouragement, inspiration, stimulus and faith in my abilities to undertake graduate study.

Sincere thanks are expressed to Mrs. D. W. Church for her cooperation and technical assistance.

TABLE OF CONTENTS

LIST OF TABLES

TABLE PAGE

LIST OF FIGURES

FIGURE PAGE

INTRODUCTION

Previous studies of animal viruses and their relationship to the host cell prompted investigation of infectious bronchitis virus in this same category.

The purpose of this research was to study the virus-cell interrelation of infectious bronchitis virus and chicken embryo kidney cells.

The objective was the adaptation of a chicken embryo-adapted strain of infectious bronchitis virus to chicken embryo kidney cells and to determine if any cytopathic effect of the virus could be used for study of the multiplication cycle.

REVIEW OF LITERATURE

Infectious bronchitis, a specific respiratory disease of chickens, is caused by a virus with an average diameter of 60 to 100 mμ (Reagan et al., 1948; Reagan et al., 1950; Reagan and Brueckner, 1952).

Infectious bronchitis virus (IBV) can be propagated readily in chicken embryos by various routes of inoculation (Beaudette and Hudson, 1937).

On primary isolation of the virus in chicken embryos, gross pathological lesions such as "curling and stunting" of the embryos, excessive urates in the kidneys, and clubbing of the down are observed. Mortality of embryos is variable (Beaudette et al., 1937; Cunningham, 1952; Cunningham and Stuart, 1947; Cunningham and Jones, 1953; Delaplane and Stuart, 1941; Fabricant, 1949; Fabricant, 1956; Loomis et al., 1950).

Serial passage increases the virulence of the virus for embryos, but decreases the virulence for chickens. The criterion of complete adaptation of the virus to embryos is the ability of the virus to kill all embryos within 24 to 36 hours. Embryo-adapted virus is nonimmunogenic but is specifically neutralized by anti-infectious bronchitis serum (Beaudette et al., 1937; Delaplane and Stuart, 1941; Fabricant, 1951; Page, 1950; Page, 1954).

Fahey and Crawley (1956) cultivated the Connaught Laboratories vaccine strain R and the Beaudette embryo-adapted strain of IBV in stationary flask cultures of fragments of minced chicken embryo kidney, liver, and chorioallantoic membrane (CAM) and in monkey kidney cells. The virus multiplied in the tissue cultures as determined by chicken embryo infectivity tests, but cytopathic effects (CPE) could not be observed in the infected cultures. These strains did not infect mouse liver cells or Hela cells (Davis, 1956, cited by Buthala and Mathews, 1957).

Buthala and Mathews (1957) were not successful in propagating the 170th embryo passage of a strain of IBV in monolayers of chicken embryo kidney cells (CEKC).

Chomaik et al. (1958) reported that CEKC may be infected with the Beaudette strain as shown by CPE. The Massachusetts and Connecticut strains did not cause infection. The 10th CEKC passage of the Beaudette strain caused CPE of a few chicken embryo fibroblasts. The CPE of the virus became more extensive with further passages in fibroblasts.

The Beaudette strain can be serially passed in whole and minced CAM suspended in Hank's balanced salt solution (BSS) and in Morgan's medium 199 (Cunningham and Spring, unpublished data; Ferguson, 1958; Hanks, 1949; Ozawa, 1959). Growth was evident in the 12th passage which represented a dilution of the virus far in excess of the

titer of the original seed virus. This indicated that an adaptive process was necessary for tissue culture as well as for chicken embryo cultures. Mortality of embryos was used as the criterion of infectivity for the CAM-passaged virus (Ferguson, 1958).

Hitchner and White (1955) also used mortality of embryos as the criterion for infectivity in growth-curve studies of the Beaudette strain. Any embryo showing one or more of the criteria of curling and stunting, clubbed down, or excessive urates was recorded as infected by the Connaught R strain.

The log phase for the Beaudette strain began four hours postinoculation. The maximum concentration was reached at 12 hours. The Connaught R strain entered the log phase in six hours, and the maximum concentration of the virus was attained within 24 to 30 hours.

Ackermann et al. (1954) demonstrated multiplication of poliomyelitis virus, Saukett strain, Type III, using Hela cell monolayers. Tube cultures containing 10^5 Hela cells were inoculated with one ml of tissue culture fluid containing $10^{7.5}$ tissue culture infective doses ($TCID_{50}$). The cultures were incubated at 37 C for one hour. The inoculum was removed, and the culture was washed five times with maintenance medium. After one ml of nutrient medium per tube was added the cultures were incubated at 37 C. At hourly intervals, 0.1 ml portions of fluid were removed

for titration in other tubes of Hela cell cultures. Each portion of fluid that was removed was replaced by the same volume of fresh medium. The latent period was nearly four hours. The release of virus during the next five to six hours was at an exponential rate. The maximum yield was reached within 10 to 11 hours. A single sequence of infection was produced in Hela cells by use of a massive inoculum of poliomyelitis virus. The CPE of the virus was used as the criterion of infectivity. The growth curve was similar to a single sequence of infection produced in cultures with influenza or Western equine encephalomyelitis (WEE) virus. Lack of synchronization of infection of individual cells was indicated by the differences in the rate at which cytopathic alterations developed in the cells.

Fahey and Crawley (1956) found in growth curve studies using stationary flask tissue cultures of minced CAM that the adsorption period for the Beaudette strain, sixth tissue culture passage was from four to eight hours. The latent period varied from 12 to 18 hours. The maximum titer was obtained in approximately 40 hours, as contrasted to 12 hours in chicken embryos. The multiplication cycle, using trypsinized monkey kidney cells, revealed that no fresh virus appeared for eight to ten hours. No CPE was evident.

Growth curve studies by Ozawa (1959) with the Beaudette strain grown in a suspension of CAM in BSS showed

the following phases of the multiplication cycle: (1) a variable lag phase of eight hours; (2) a logarithmic phase during the next 52 hours; (3) a primary decline phase over the following 60 hours; (4) a stationary phase of 72 hours; (5) a secondary decline phase of 48 hours.

The Beaudette strain cultivated in CEKC was used by Chomiak et al. (1958) in growth-phase studies. Titrations in the cell cultures were used with CPE as the infectivity response of the virus. The virus disappeared from the extracellular fluid within four hours, reappeared in 16 hours, and reached a maximum titer at 48 hours, where it remained for 24 hours and then declined. When titrated in chicken embryos, the titer of the CEKC-adapted virus was 10^3 higher than when titrated in CEKC. No statement was made as to the cell passage of virus used, but it may be assumed that it was in one of the first 10 CEKC passages.

In addition to the microscopic examination of infected cell cultures for CPE as an indication of viral infectivity, certain animal viruses may be assayed by the plaque technic.

Wright and Sagik (1958) reported plaque formation by the Beaudette strain in CEKC. Plaques were apparent in 16 to 18 hours. After a three-day incubation period, the plaques were three to four mm. in diameter and could be seen without the aid of the microscope before staining. Titers obtained by the plaque count (PFP) and by embryo

infectivity showed a ratio of four when it was assumed from the Poisson distribution that one embryo lethal dose$_{50}$ equals 0.7 virus particle.

Dulbecco and Vogt (1954) investigated the rate of adsorption of WEE virus on whole chicken embryo cells by the plaque technic. One-step growth curves were determined in cell suspensions and on cell layers. The latent period was shorter with the cell layer and varied between two and three and one half hours, followed by an initial exponential rise reaching a maximum after six to eight hours.

Rubin et al. (1955) used the plaque technic to study the intracellular appearance and release of WEE virus in suspensions of infected chicken embryo fibroblasts. No intracellular virus was found during the first hour after adsorption, indicating that the virus was non-infectious after entering the susceptible cell. The first progeny of the virus was detected in the cells between one and two hours after infection and increased in amount exponentially during the next three hours. The released virus in the extracellular fluid increased at the same rate, but exceeded the intracellular virus by a factor of 20 during the period of the exponential increase. The authors concluded that a virus particle could be released from the cell within one minute after infection.

Howes and Melnick (1957) inoculated monolayers of monkey kidney cells with poliomyelitis virus, Brunhilde

type, for studies of the maturation and release of the virus in a single growth cycle. At various intervals the virus in the cell monolayer, cell-associated virus (CAV), and "free" virus in the medium was assayed by the plaque technic. An accurate picture of virus maturation in this system was obtained by considering that the total virus was composed of CAV plus "free" virus. The virus was produced at a slow rate, and only 50 per cent of the total amount of virus appeared within four hours and 48 minutes to six hours and 12 minutes. This is in contrast to WEE virus in chicken embryo fibroblasts where the virus was released rapidly, and "free" virus was considered to be an approximation of the "total" virus.

Levine and Sagik (1956) demonstrated a one-step growth curve of Newcastle disease virus (NDV) on whole chicken embryo monolayers by the plaque technic. The latent period of three to four hours was followed by a log period which lasted from four to eight hours.

Rubin and Franklin's (1957) experiments assaying NDV by the plaque technic using chicken embryo lung cells determined the velocity constant for adsorption and multiplication of the virus. "Free" virus and CAV were equal in amount during the period of the exponential increase of virus. The apparent release time was calculated to be about 80 minutes.

Howes (1959 A) studied the maturation and release

of poliomyelitis virus, Type I, in a suspended cell population of monkey kidney cells using the plaque technic for estimating the total virus and "free" virus. The amount of "free" virus appearing during the maturation phase with suspended cells was more than that in cell monolayers, but the growth cycles were essentially the same (Howes and Melnick, 1957). The rate of release of the virus was much less than the rate of maturation. A large proportion of the virus yield accumulated during maturation within the cell. The maturation phase began four hours after the virus entered the cell. The virus release began within 30 to 60 minutes after the beginning of maturation.

Howes's (1959 B) work on poliomyelitis virus, Type I, from a single Hela cell, contributed information on the variation in time when the first mature virus was detected. A single cell released virus during one hour or less. In multiple cell populations, the maturation and release phases of the growth cycle in individual cells overlapped. In a single cell, these phases were separated by an intracellular retention phase which varied from a few minutes to an hour.

MATERIALS, METHODS AND RESULTS

Preparation of Chicken Embryo Kidney Cells

Primary cultures of CEKC were used throughout the study. The kidneys from 16-day-old chicken embryos were removed aseptically and washed in Hank's BSS in a Petri dish. The kidneys were minced finely into pieces about 0.5 to 1.0 mm^3, which were then thoroughly washed in BSS. The pieces were transferred to a 250 ml, fluted Erlenmyer flask containing a "Teflon" covered magnet, and 100 ml of Seitz-filtered 0.25 per cent trypsin at pH 8.4 pre-warmed to room temperature. The flask was placed over a "Magnestir," and trypsinization was performed at room temperature for one hour.

The cell suspension was filtered through eight layers of cheesecloth and centrifuged at 1500 rpm (437 x G) for five minutes in an International PR-1 centrifuge at four C. The supernatant fluid was discarded, and the packed cells were resuspended in BSS. This procedure of centrifugation and washing of the cells was repeated three times. The packed cells from the last washing were diluted 1:400 in a growth medium consisting of 0.5 per cent lactalbumin hydrolysate in BSS, 10 per cent bovine serum, 100 units of penicillin and 0.1 mg. of streptomycin. A 1:400 dilution

of cells averaged about 950,000 to 1,000,000 cells per ml as determined with a hemacytometer. Leighton tubes, 16 x 125 mm, were seeded with 1 ml of the cell suspension per tube, sealed with white stoppers, and incubated at 37 C in the horizontal position.

It was essential that the Leighton tubes be clean without traces of cleaning compound or without old cell cultures adhering to the walls of the tube. The tubes were cleaned in acid cleaning solution, rinsed eight times in tap water, and 12 times in distilled water.

At 24 hours when the monolayer of cells was formed, the growth medium was replaced with 1 ml of a maintenance medium consisting of 0.5 per cent lactalbumin hydrolysate in BSS and two per cent bovine serum. The monolayer was then inoculated with 0.2 ml per tube of the Beaudette embryo-adapted strain, North Central Infectious Bronchitis Virus Repository, Code 42. This strain was chosen because it has the ability to kill chicken embryos within 36 hours post-inoculation via the allantoic cavity, which would serve as a means of assay of viral infectivity of the extracellular fluid from chicken embryo kidney cells.

A definite routine was established and followed throughout the experiments. Trypsinized CEK primary cells were prepared and seeded. The growth medium was replaced by maintenance medium and the culture was inoculated 24 to 28 hours after seeding.

Microscopic observations of the infected monolayer were recorded 48 hours post-inoculation. The extracellular fluid was decanted from the tubes and pooled. The pooled sample was distributed in screw cap vials, 3 ml per vial, and stored at -60 C. Five days later the virus-infected fluid was thawed and was used as inoculum on new primary cells.

Adaptation of IBV to Chicken Embryo Kidney Cells

The first passage of IBV in CEKC was made by inoculating each of three tubes with 0.2 ml of virus-infected allantoic fluid containing 10^6 embryo infective doses per 0.1 ml. Microscopic examination of the cells 24 hours after inoculation revealed no apparent CPE produced by the virus. The extracellular fluid was harvested, pooled, and used to inoculate three more tubes.

On the second passage, CPE was evident 48 hours after inoculation. The cell monolayer was not uniformly affected, but it was evident that infection of the cells had occurred.

The affected cells were rounded and clumped together. The periphery of the cells was dense, and the cytoplasm was clear without inclusion bodies or granules. Degeneration and necrosis were the over-all picture. In some areas of the monolayer, the dead cells had sloughed

from the wall of the tube. There was a tendency for the dead cells to aggregate (figures 1, 2, 3 and 4).

By the fifth passage, the virus was well adapted to propagation in CEKC. The entire monolayer showed evidence of viral infection. The virus was serially passaged 55 times.

Titration of Virus in Chicken Embryo Kidney Cells

The fifth cell culture passage of the virus was used for the first titration of viral infectivity using CPE as the positive response.

Serial ten-fold dilutions of the virus were prepared using as the diluent 4.5 ml of BSS containing 0.5 per cent lactalbumin hydrolysate. The virus was thawed and 0.5 ml was transferred to the first tube with a 2 ml serological pipette. With another 2 ml pipette, the contents of the tube were mixed by aspirating and expelling the mixture from the pipette 20 times, and then transferring 0.5 ml to the next tube. This was continued until all dilutions were made. The virus dilutions were kept in an ice bath to prevent inactivation of the virus at room temperature. Each dilution of virus was used to inoculate three tubes of cell culture, 0.2 ml per tube, which were incubated at 37 C for 48 hours when microscopic examinations were made.

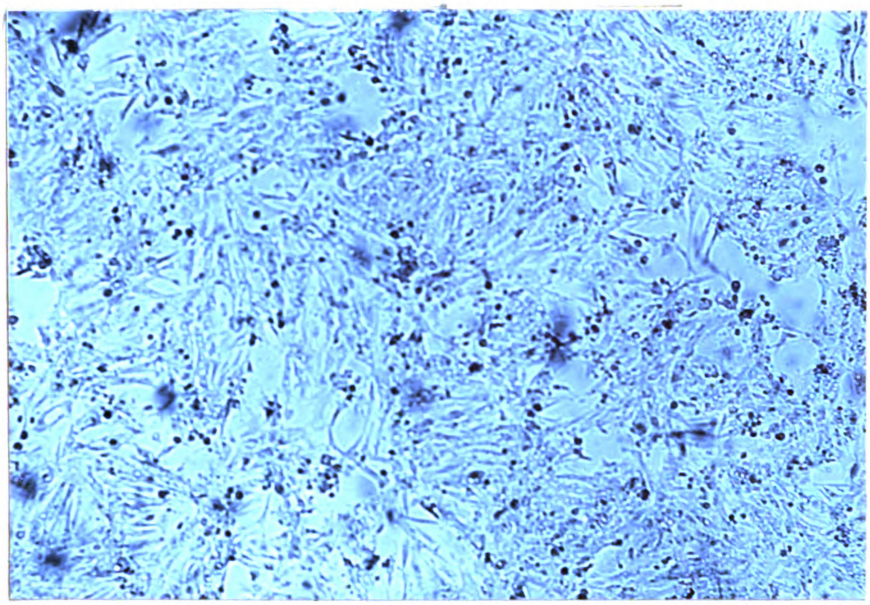

Figure 1. Normal chicken embryo kidney cells (x 100).

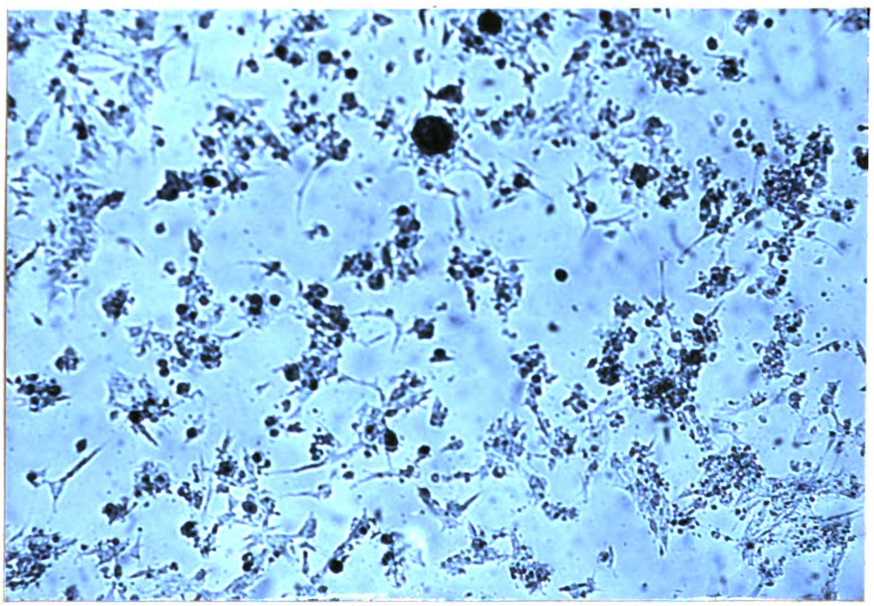

Figure 2. Chicken embryo kidney cells infected with the 54th cell passage of chicken embryo kidney cell-adapted infectious bronchitis virus (x 100).

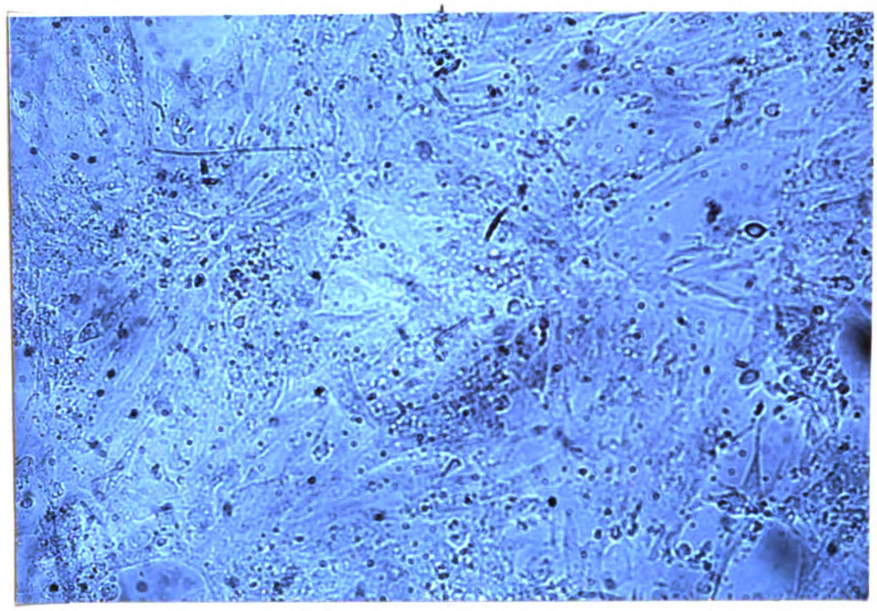

Figure 3. Normal chicken embryo kidney cells (x 200).

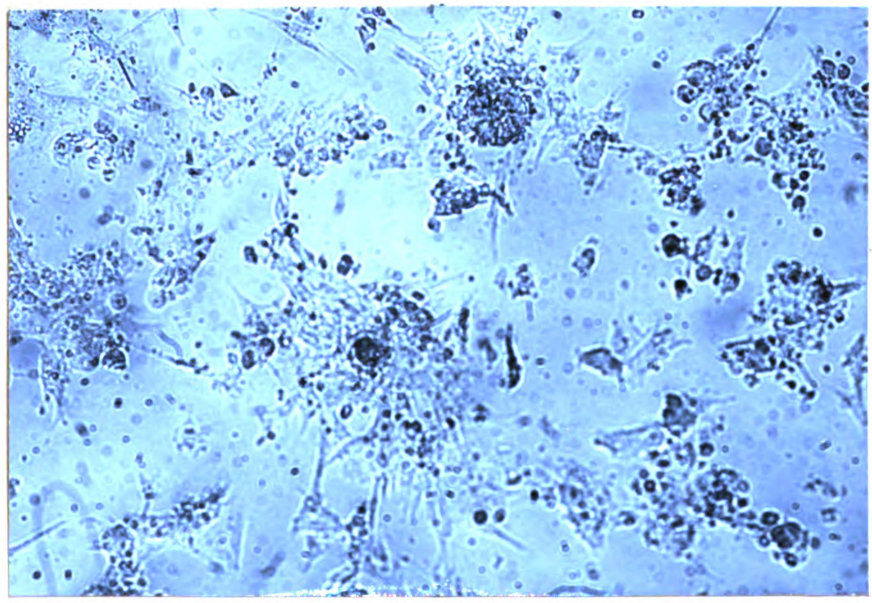

Figure 4. Chicken embryo kidney cells infected with the 54th cell passage of chicken embryo kidney cell-adapted infectious bronchitis virus (x 200).

The endpoint of viral infectivity was considered to be the highest dilution of the virus in which CPE was present in at least two of the three tubes. The titer of the virus was the reciprocal of the end point dilution.

The titer of the fifth passage of the virus was 10^3 /0.2 ml in cell culture at least but 10^5 /0.2 ml in chicken embryos.

As the virus became adapted to CEKC in subsequent passages, the titer was higher when assayed in cells than in embryos. At the 55th passage, the titer in CEKC was 10^9 or higher, while in chicken embryos the titer was only 10^5. As shown in figure 5 there was a general increase of viral infectivity related to cell culture passage as compared to a rather constant level as determined by titration in chicken embryos.

Neutralization Tests

Decreasing virus-constant serum method

Serial tenfold dilutions of the CEKC-passaged virus were prepared using the procedure previously described. Undiluted, immune and normal chicken sera were filtered through Swinney Seitz filters and inactivated at 56 C for 30 minutes. One part of the serum, 0.3 ml, was mixed with one part of the respective virus dilutions, and 0.2 ml of the serum-virus mixture was used to inoculate each of three

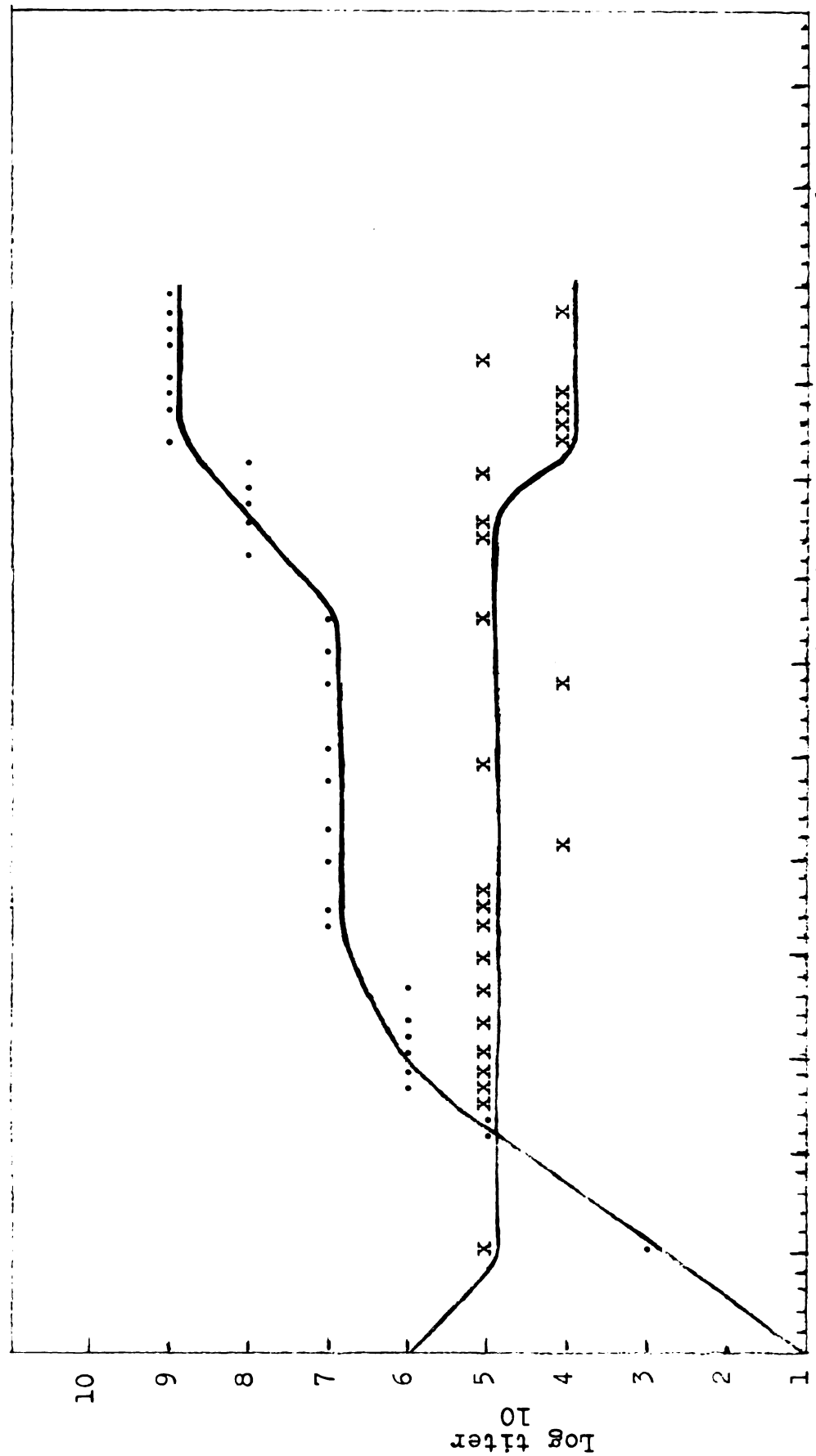

Figure 5. Infectivity of Chicken Embryo Kidney Cell-Adapted Infectious Bronchitis Virus at Different Passages as Titrated by Cell and Embryo Response

• Titration in chicken embryo kidney cells
x Titration in chicken embryos

tubes per dilution. The virus was also titrated, using three tubes per dilution.

The reciprocal of the difference between the endpoints of infectivity for the virus and the virus-serum mixtures was the neutralization index for the serum.

When undiluted immune and normal sera were used, a precipitate formed on the cell monolayer and prevented an accurate assessment of CPE. With the sera diluted 1:10, the precipitate was partially eliminated, and the CPE could be determined.

Constant virus-decreasing serum method

Serial tenfold dilutions of the serum were prepared. Each serum dilution, 0.3 ml, was mixed with an equal part of virus containing a certain number cell culture doses (CCD), and 0.2 ml of the mixture was used for the inoculum per tube.

The endpoint of viral infectivity of the serum-virus mixture was considered to be the highest dilution of the serum in which not more than one of the three tubes showed CPE. The neutralization index of the serum was the reciprocal of the endpoint dilution.

When the decreasing virus-constant serum method for neutralization was used, the NI was 10^8 for anti-IBV serum with the 44th and 45th passages of cell-cultured virus. An NI of 10^2 was obtained with embryos using the

same cell-passages.

When the 41st through the 45th CEKC-passages of virus was used, and the same antiserum for each test, each of which was performed at a different time, it was found that when 10^7 CCD were used the endpoint dilution of neutralization was 10^1. With 10^5 CCD the endpoint was 10^2; 10^4 CCD, 10^3; and 10^4 and 10^2 CCD, 10^3. With both methods for determination of neutralizing antibody, the normal sera were negative (table 1).

Multiplication Cycle in Chicken Embryo Kidney Cells

Studies of the adsorption of the cell-adapted virus to the cells, intracellular multiplication, and subsequent release of new virus were performed by the following method.

The maintenance medium was decanted from a group of tubes containing a 24 hour monolayer of CEKC, and each tube was inoculated with 0.2 ml of a certain number of CCD of the virus. The tubes were incubated at 37 C in the horizontal position to insure that the inoculum completely covered the monolayer of cells. At 5, 10, 15, 20, 30, 40, 45, 50, 60, and 75 minutes, three tubes were randomly selected. The inoculum was removed from each tube with a Pasteur pipette. The monolayer was then washed three times with BSS, before adding one ml of maintenance medium

TABLE 1

Neutralization tests in cell culture

Decreasing virus--constant serum

CEKC Pass. of virus	Titer of virus	Neutralization endpoint dilution of serum	NI
11	10^6	undiluted	Not readable
44	10^8	10^1*	10^8
45	10^8	10^1*	10^8

* Serum diluted 10^1

Constant virus--decreasing serum

CEKC Pass. of virus	CCD of virus used	Neutralization endpoint dilution of serum
12	10^4	10^2
13	10^5	10^2
26	10^6	10^2
41	10^7	10^1
42	10^5	10^2
43	10^2	10^3
44	10^4	10^3
45	10^4	10^4

per tube. The tubes were incubated for 48 hours at 37 C.

This experiment was to determine the time interval and the number of CCD to be used for adsorption and for determination of the virus subsequently released from the cells.

Adsorption of the virus to the cells was considered to have taken place when the typical CPE pattern of viral infectivity was present.

The virus-cell attachment occurred within five minutes, using from 10^1 CCD to 10^7 CCD/0.2 ml as inoculum. The time for adsorption did not appear to be related to the number of CCD or to the cell passage level of the virus (table 2).

An adsorption period of 20 minutes using 10^5 CCD of the virus was arbitrarily chosen to study the time of release of the virus from the infected cell. For this portion, three tubes were inoculated with 0.2 ml of virus and incubated at 37 C. The infected fluid was then removed, and the tubes were handled the same as for the preliminary studies on adsorption. The BSS from the third washing was pooled and placed in an ice bath for titration within the next one to two hours. Growth medium, one ml per tube, was added to the three tubes, which were incubated at 37 C for five minutes.

Samples from the three tubes were collected, pooled, and placed in the ice bath, for titration of virus

TABLE 2

Adsorption of virus on chicken embryo kidney cells

CEK Pass. No. of Virus	CCD of Virus	Adsorption Time in Minutes									
		5	10	15	20	30	40	45	50	60	75
11	10^4	...	...	+	...	+	...	+	...	+	+
15	10^3	...	+	...	+	+	+	...	+	+	...
46	10^1	+	...	+	...	+	...	...	...	...	...
46	10^3	+	...	+	...	+	...	...	...	...	...
46	10^5	+	...	+	...	+	...	...	...	...	...
46	10^7	+	...	+	...	+	...	...	...	...	...

released within five minutes. The monolayer was washed again three times with BSS, and the last wash pooled and placed in the ice bath for titration. Growth medium, one ml per tube, was again added to the three tubes which were incubated at 37 C for an additional 15 minutes. This process was continued for one, two, three, six, fourteen hour intervals. The complete procedure is outlined in figure 6.

Titration of the free virus released and of the pool of the last BSS washing was performed as previously described.

The extracellular virus decreased to 10^1 during the eclipse phase of five minutes and increased to a maximum titer of 10^9 during the 25 minute log phase. The titer remained constant during a stationary phase of 60 minutes. It then gradually declined to 10^2 during the next five hours and remained constant for the following 14 hours (figure 7). The final washing from the cells at each sampling period was noninfectious.

Storage of CEKC-Adapted IBV

The viability of the virus for varying time periods was determined.

Different passages were chosen at random and were used to inoculate 24 hour CEKC monolayers and 10-day-chicken embryos, 0.2 ml inoculum per tube and per egg.

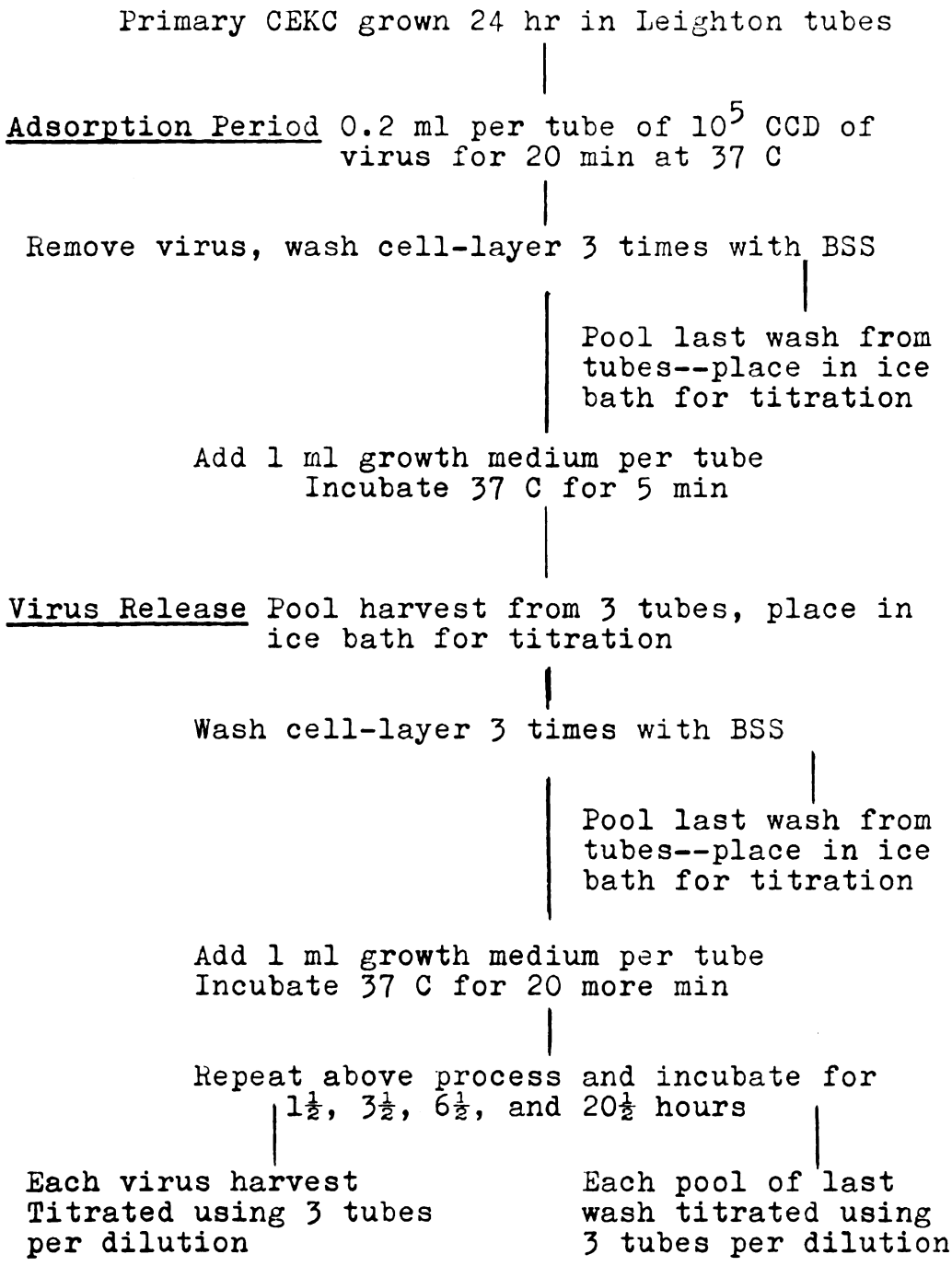

Figure 6. Procedure for study of the adsorption of chicken embryo kidney cell-adapted virus to the cells and release from the cells.

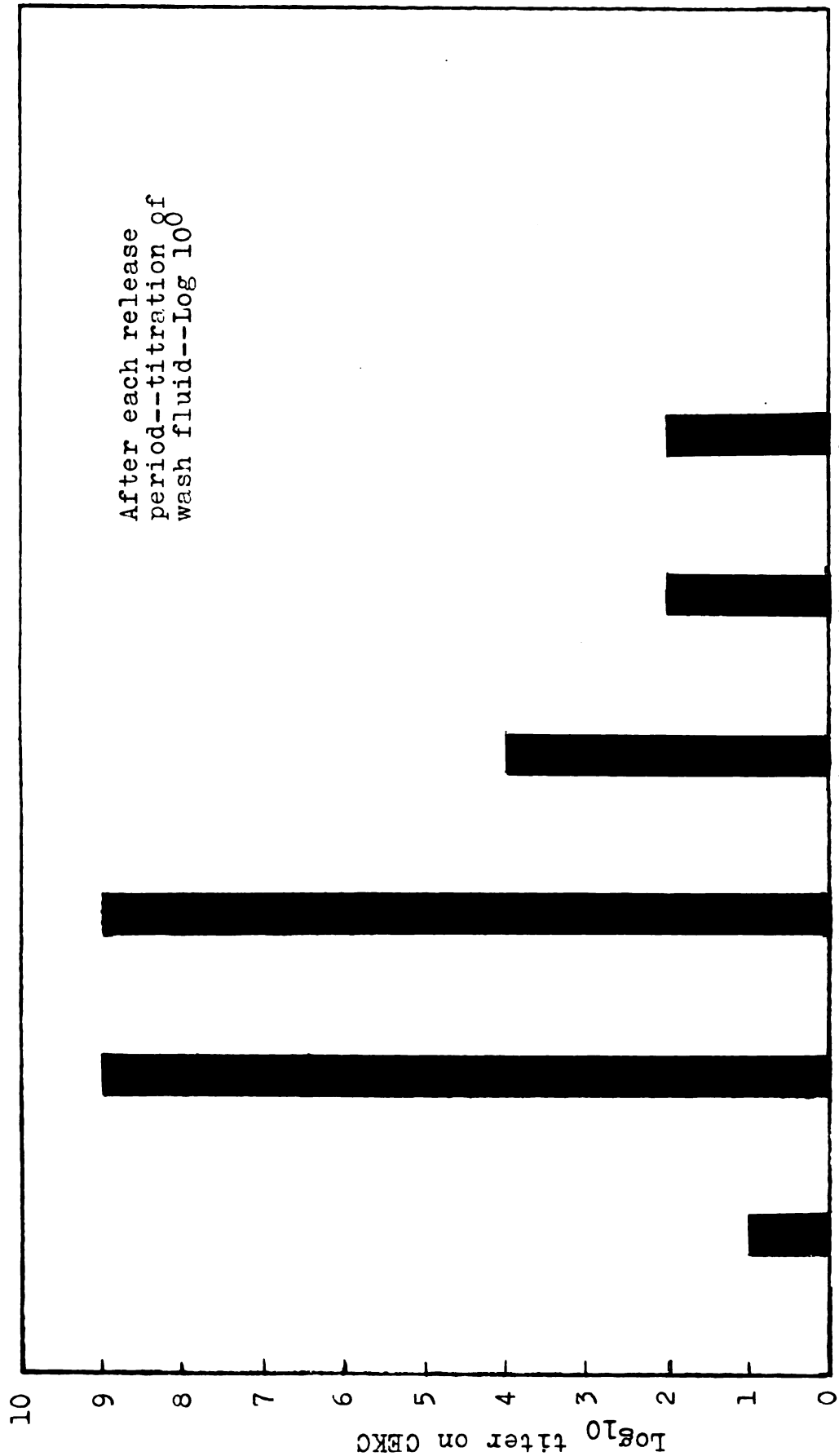

Figure 7. Multiplication cycle of chicken embryo kidney cell-adapted infectious bronchitis virus.

The CPE of the virus on the cells and the mortality of the embryos were the criteria of infectivity of the virus (table 3). The virus remained viable for at least 274 days, the longest test period, at -30 C.

TABLE 3

Effect of storage on chicken embryo kidney cell-adapted IBV

Cell Pass. No.	Temp.	Time in days*	CPE	Embryo Mortality
6	-30 C	274	+	5/5
9	-30 C	268	+	5/5
11	-30 C	260	+	5/5
14	-30 C	240	+	5/5
21	-30 C	184	+	5/5
27	-62 C	135	+	5/5
31	-62 C	92	+	5/5
34	-62 C	53	+	5/5
38	-62 C	18	+	4/5

* The survival times are not to be considered the maximum period of viability because the later passages had not been stored for longer periods.

DISCUSSION

A virus which has an affinity for and is apparently restricted to a specific host will multiply and induce the disease in the host. Early investigators were concerned with the phenomenon of recognizing the presence of a virus by the signs and lesions which were produced in an animal. However, it is now evident that many animal viruses can be adapted to and multiply in a variety of hosts such as laboratory animals, embryonating chicken eggs, and cell and tissue cultures of animal origin.

The concept has been established that any microbiological agent in order to grow and proliferate must orient itself to a new environment. A virus must become accustomed to a new environment before signs of infection, specific lesions, or a growth pattern can be recognized. It is possible that infection may have occurred without being recognized on the initial passage of the virus in a new host.

Infectious bronchitis is a specific respiratory disease of chickens, and the adaptation of the causal virus to chicken embryos was relatively uncomplicated. The process of adaptation was accomplished through serial passage of the virus in the new host. Definite signs of infection in embryos may be observed on the first passage, but in

some instances two or three passages may be necessary before gross evidence of infection may be detected. Adaptation was considered to be complete when all embryos were killed. Embryo cultured virus capable of infection does not revert to its original characteristics after administration to chickens.

The presence of excessive urates in the chicken embryo kidney was evidence that the kidney cells were infected by the virus. Assuming that these cells have a definite reception for IBV, adaptation of the virus to them would offer host cells in which to produce virus. If IBV could be adapted to cell culture and if signs of infection were present, many studies of the virus-cell interrelation could be performed.

It is well known that a virus is dependent upon the host cell to provide the essential metabolites and enzymes which it lacks for independent growth and multiplication. One could assume that during the first passage of the embryo-adapted strain of IBV in CEKC, a period of adjustment would be required. The virus would have to become accustomed to the metabolism of the cells, the medium for the cells, and the environmental influences. If the conditions were suitable, some of the virus particles would survive and would multiply selectively in the new cell. The virus particles which survive and produce new progeny would have a higher population rate per cell

and would show evidence of a more lethal nature. Subsequent serial passage of the virus would result in a stable population of virus adapted to the cell.

Another theory might be that the cell itself was more easily penetrated by the virus after the virus had been exposed to the new environment. After the adaptation had been established, the CPE of the virus could be used as the positive response for infectivity.

The relationship between the amount of the virus-infected fluid and the response of the cell was the basis for titration of the CEKC-adapted strain on CEKC and in chicken embryos. Adaptation of IBV to chicken embryo culture following isolation of the virus from the natural host and the adaptation of embryo cultured virus to CEKC have a parallelism in that the infectivity of the virus increases on serial passage in the new host. This would appear to be a normal process in which the virus became more lethal to the kidney cells than to the entire chicken embryo. The virus would be able to react directly with the cell in a defined medium, in contrast to the many tissues and organs of the chicken embryo. The defense mechanisms within the embryo and embryonic fluids are factors to be considered as well as the influence of the metabolism of the embryo.

Chomiak's et al. (1958) data on the titration of the Beaudette embryo-adapted strain to CEKC showed the

infectivity titer to be 10^3 higher in chicken embryos than in CEKC. This is the reverse of the results reported in the present data. When IBV was cultivated in the CAM suspended in BSS, the CAM-adapted virus had a higher titer than the original seed virus when titrations were made in chicken embryos. This would support the hypothesis that successful adaptation of IBV to a new host is accompanied by increased lethality of the virus.

The infectivity of the CEKC-passaged virus was neutralized by anti-IBV serum using chicken embryos and CEKC for indication of the response.

Burnet et al. (1937) hypothesized that viral inactivation by immune serum was the result of a reversible union of antibody with the surface of the virus. Inactivation was the result of the interaction between the susceptible cell and antibody-coated viral particle.

Dulbecco's et al. (1956) theory of the neutralization process was that a direct irreversible combination of virus and antibody molecule occurred independent of the cell system, and was linearly dependent on the concentration of the antibody. The kinetics of neutralization in the presence of excess antibody was of a first order reaction indicating that one molecule of antibody was responsible for neutralization of one infective unit of virus.

Fazekas de St. Groth et al. (1958 A, 1958 B) assumed that the antigen-antibody reaction was reversible and that

the dissociable complexes were formed by the union of the antibody molecule and the antigenic sites on the virus.

With the constant virus-decreasing serum method, precise determinations of the ratio of neutralizing antibody and antigen were not made as the primary purpose was to establish that neutralization had occurred. The results obtained, however, indicate that there is a relation between the amount of virus used and the degree of neutralization. The data presented indicate a proportionality but are not sufficiently accurate, due to several variables, to substantiate definite conclusions. It would be anticipated that a proportionality of antigen and antibody would occur with IBV similar to other animal viruses and should be a fruitful area for further investigation.

It is not known how animal viruses penetrate a cell wall. Viruses of some groups adsorb rapidly to the host cells. The CEKC-adapted strain of IBV attached to the cell within five minutes. The equally rapid release of the infectious viral particle from the susceptible cell into the extra-cellular fluid suggests that the maturation process occurs near the periphery of the cell. It can be assumed from these results that most of the reaction is completed during a single phase, and the production of successive infective units is not involved in the IBV multiplication cycle. This follows a general occurrence in infection of cells by viruses where the pattern is one in

which there is an eclipse phase where little or no extracellular virus can be recovered. Nonrecoverability of the virus soon after inoculation has been reported for rabies, poliomyelitis, St. Louis encephalitis, yellow fever, influenza, and several other viruses. The eclipse phase is followed by log, stationary, and decline phases.

Infectious bronchitis virus, like WEE and influenza virus, presents an explosive type of reaction. A short adsorption period is followed by a rapid release of the infectious particle into the extracellular fluid. In contrast, poliomyelitis and Rous sarcoma viruses release into the extracellular fluids more slowly. In order to obtain an accurate picture of the maturation of poliomyelitis virus, the assay must include CAV and the "free" virus.

In the studies of the adsorption and release of IBV, the total virus was considered to be approximately that of the virus assayed from the extracellular fluid. Titration of the final BSS wash fluid after each collection period revealed that the cells were washed free of virus and would not be reinfected from extracellular virus.

Chomiak et al. (1958) inoculated cultures with adapted virus and measured the cumulative production of virus by removing portions of the extracellular fluid and by replacing it with equal amounts of medium. The virus disappeared from the fluid after four hours and reappeared

at the 16th hour. The maximum infectivity was attained at 48 hours and remained constant until 72 hours.

A comparison of the growth cycles by these different methods of determination of virus adsorption and release from the cell is not feasible. A more accurate picture of the amount of virus released in a given time interval is obtained by removing all the extracellular fluid from the cells for titration rather than only portions of the fluid. Washing the cells free of virus prevents reinfection of the cells and presents a more accurate estimate of the actual virus released from the cell. However, the same general pattern of multiplication and release of IBV is followed.

The infectious bronchitis virus-cell interaction is definitely explosive and reflects the type of infection as observed from signs of the natural or experimental disease in the chicken.

SUMMARY

1. The Beaudette embryo-adapted strain of infectious bronchitis virus was propagated for 55 serial passages in primary monolayers of chicken embryo kidney cells.
2. The cytopathic effect of the virus was used as the criterion of infectivity.
3. The titer of the virus increased with adaptation by serial passage in cell culture. After about the 15th cell passage, the titer as determined with chicken embryo kidney cells was about 10^4 or 10^5 higher than when titrated in chicken embryos.
4. The cell-passage of IBV was neutralized by anti-infectious bronchitis sera. Normal sera did not neutralize the virus.
5. The relation between the amount of virus used and degree of neutralization indicated a proportionability between the ratio of antibody and antigen.
6. Adsorption of the cell-adapted virus to cells occurred within five minutes or less.
7. Release of the virus from the cell into the extracellular fluid was equally rapid. The maximum titer was reached at one and one half hours after inoculation of the cells.
8. A typical multiplication pattern of a lag phase, log

phase, stationary phase and gradual decline phase occurred.

9. Cell-cultured virus remained viable at -30 C for at least 274 days.

BIBLIOGRAPHY

Ackermann, W. W., Robson, A., and Kurtz, H. 1954 Growth characteristics of poliomyelitis virus in Hela cell cultures. J. Exptl. Med. 100, 437-450.

Beaudette, F. R., and Hudson, C. B., 1937 Cultivation of the virus of Infectious bronchitis. J.A.V.M.A., 90, 51-58.

Buthala, D. A., and Mathews, J., 1957 Use of cellular cultures of chicken embryo kidney tissue in virus studies. Cor. Vet. 47, 143-159.

Burnet, F. M., Keogh, E. V., and Lush, D., 1937 The immunological reactions of the filterable viruses. Australian J. Exptl. Biol. Med. Sci. 15, 266-368.

Cunningham, C. H., 1952 Methods employed in the diagnosis and investigation of Infectious bronchitis and Newcastle disease. Proc. Book, A.V.M.A., 250-257.

Cunningham, C. H., 1957 A Laboratory Guide in Virology. 3rd. edition, Burgess Publishing Co., Minneapolis, Minn.

Cunningham, C. H., and Spring, M. P., unpublished data.

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

Cunningham, C. H., and Jones, M. H., 1953 The effect of different routes of inoculation on the adaptation of Infectious bronchitis virus to embryonating chicken eggs. Proc. Book, A.V.M.A., 337-342.

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

Davis, R. N. 1956 Attempts to propagate Infectious bronchitis virus in tissue cultures of embryonic chicken hearts and Hela cells. M.S. thesis, A and M College of Texas. Cited by Buthala and Mathews 1957.

Delaplane, J. P., and Stuart, H. O., 1941 The modification of Infectious bronchitis virus of chickens as the result of propagation in embryonated chicken eggs. Rhode Island State Coll., Agric. Exper. Sta. Bull. 284.

Dulbecco, R., and Vogt, M., 1954 One-step growth curve of Western Equine Encephalomyelitis Virus on chicken embryo cells grown in vitro and analysis of virus yields from single cells. J. Exptl. Med. 99, 183-199.

Dulbecco, R., Vogt, M., and Strikland, A. G. R., 1956 A study of the basic aspects of neutralization of two animal viruses Western Equine Encephalitis Virus and poliomyelitis virus. Virology 2, 162-205.

Fabricant, J., 1949 Studies on the diagnosis of Newcastle disease and Infectious bronchitis. II The diagnosis of Infectious Bronchitis by virus isolation in chick embryos. Cornell Vet., 39, 414-431.

Fabricant, J., 1951 Studies on the diagnosis of Newcastle disease and Infectious bronchitis of fowls. IV The use of the serum neutralization test in the diagnosis of Infectious bronchitis. Cornell Vet., 41, 68-80.

Fabricant, J., 1956 A modified chick embryo inoculation technique for the isolation of viruses from the respiratory tract of chickens. Proc. Northeast Conf. Lab. Workers Pullorum Dis. Control, Univ. Delaware, Newark.

Fahey, J. E., and Crawley, J. J., 1956 Propagation of Infectious bronchitis virus in tissue culture. Canad. J. Micro., 2, 503-510.

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

Fazekas de St. Groth, S., Watson, G. S., and Reid, A. F. 1958 B The neutralization of animal viruses. I. A model of virus-antibody interaction. J. Immunol. 80, 215-225.

Ferguson, G. H., 1958 Growth of Infectious bronchitis virus in suspended chorioallantoic membranes. M.S. thesis, Michigan State University.

Hanks, J. H., An Introduction to Cell and Tissue Culture. Tissue Culture Course, Cooperstown, New York, 1949-1953.

Hitchner, S. B., and White, P. G., 1955 Growth-curve studies of chick embryo-propagated Infectious bronchitis virus. Poult. Sci., 34, 590-594.

Howes, D. W., 1959 A The growth cycle of poliovirus in cultured cells, II. Maturation and release of virus in suspended cell populations. Virology 9, 96-109.

Howes, D. W., 1959 B The growth cycle of poliovirus in cultured cells III. The asynchronous response of Hela cells. Multiply infected with Type I poliovirus. Virology 9, 110-126.

Howes, D. W., and Melnick, J. L. 1957 The growth cycle of poliovirus in monkey kidney cells I. Maturation and release of virus in monolayer cultures. Virology 4, 97-108.

Levine, S., and Sagik, B. P. 1956 The interaction of Newcastle disease virus (NDV) with chick embryo tissue culture cells: attachment and growth. Virology 2, 57-68.

Loomis, L. N., Cunningham, C. H., Gray, M. L., and Thorp, F., Jr. 1950 Pathology of the chicken embryo infected with infectious bronchitis virus. Am. J. Vet. Res., 11, 245-251.

Ozawa, Y. 1959 Some properties of Infectious bronchitis virus propagated in the isolated chorioallantoic membrane. Ph. D. thesis, Michigan State Univ.

Page, C. A. 1950 Antibody response of chickens to infectious bronchitis virus. M.S. thesis, Michigan State Univ.

Page, C. A. 1954 A study of the serum neutralization test for infectious bronchitis of chickens. Ph.D. thesis, Michigan State University.

Reagan, R. L., Hauser, J. E., Lillie, M. G., and Craige, A. H., Jr. 1948 Electron micrographs of the virus of Infectious bronchitis of chickens. Cornell Vet. 38, 190-191.

Reagan, R. L., Brueckner, A. L., and Delaplane, J. P. 1950 Morphological observations by electron microscopy of the viruses of Infectious bronchitis of chickens and the chronic respiratory disease of turkeys. Cornell Vet., 40, 384-386.

Reagan, R. L., and Brueckner, A. L. 1952 Electron microscopic studies of four strains of Infectious bronchitis virus. Am. J. Vet. Res., 13, 417-418.

Rubin, H., Baluda, M., and Hotchkin, J. E. 1955 The maturation of Western equine encephalomyelitis virus and its release from chick embryo cells in suspension. J. Exptl. Med., 101, 205-212.

Rubin, H., Franklin, R. M., and Baluda, M. 1957 Infection and growth of Newcastle disease virus (NDV) in cultures of chick embryo lung epithelium. Virology 3, 586-600.

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