THE EFFECT OF NEWCASTLE DISEASE VIRUS ON SUSCEPTIBLE AND RESISTANT TISSUES CULTURED FROM CHICKENS AND RABBITS

Thesis for the Degree of Ph. D.

MICHIGAN STATE COLLEGE

Lenore Jones

1954

THESIS

This is to certify that the

thesis entitled

The Effect of Newcastle Disease

Virus on Susceptible and Resistant

Tissues Cultured from Chickens and Rabbits

presented by

Agnes Lenore Jones

has been accepted towards fulfillment of the requirements for

Ph. D. degree in Bacteriology

W. N. Mack
Major professor

Date May 21, 1954

O-169

THE EFFECT OF NEWCASTLE DISEASE VIRUS ON SUSCEPTIBLE AND RESISTANT TISSUES CULTURED FROM CHICKENS AND RABBITS

By

Agnes Lenore Jones

A THESIS

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

DOCTOR OF PHILOSOPHY

Department of Bacteriology and Public Health

1954

THESIS

11-17-54
g

ACKNOWLEDGEMENTS

The author wishes to express her sincere thanks to Dr. W. N. Mack, of the Department of Bacteriology and Public Health, under whose guidance and supervision this investigation was undertaken.

The author also wishes to express appreciation to Dr. H. J. Stafseth, of the Department of Bacteriology, and to Dr. D. T. Ewing and Dr. C. A. Hoppert of the Department of Chemistry for their helpful suggestions and criticisms of this thesis.

Sincere appreciation is also extended to the typist, Mrs. Ardeth Frisbey and to the fellow students and members of the staff of the Departments of Bacteriology and Pathology whose cooperation and encouragement made this study possible.

9946

THE EFFECT OF NEWCASTLE DISEASE VIRUS ON SUSCEPTIBLE AND RESISTANT TISSUES CULTURED FROM CHICKENS AND RABBITS

By

AGNES Lenore Jones

AN ABSTRACT

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

DOCTOR OF PHILOSOPHY

Department of Bacteriology and Public Health

1954

Approved W. N. Mack

In these experiments a comparative study has been made on the effect of Newcastle Disease Virus (N.D.V) on susceptible and resistant tissues cultured from chickens and rabbits.

Embryonating chicken, adult chicken lung, liver and spleen and adult rabbit lung, liver, spleen and lymph node tissues were cultured. Both normal and immune adult chickens and rabbits were used. Hemagglutination and infectivity tests were carried out to determine the growth curves of N.D.V. in embryonating chicken tissues.

The growth cycle was composed of (a) a lag phase of 24 hours, (b) a phase of rapid virus multiplication in which peak infectivity titers were reached in 36 hours and peak hemagglutination titers in 36 to 48 hours, (c) a plateau lasting 48 to 60 hours, (d) a sudden decrease in the rate of virus production 96 hours after N.D.V. inoculation.

Growth curves prepared from hemagglutination titrations on the tissues cultured from normal adult chickens and rabbits were similar to those of the embryonating chicken tissue but the peak hemagglutination titers occurred 36 to 96 hours after N.D.V. inoculation. This was also found in the immune chicken lung and spleen cultures but not in the liver cultures. In the immune chicken liver tissues and in all the tissues of the immune rabbit there was a steep decrease in infectivity titers from an LD_{50} of 10^{-7} on the day of inoculation to 10^{-1} or 10^{-2} 48 hours later. Hemagglutination activity was recovered only in the undiluted fluids

from the immune chicken liver cultures.

Cytological degeneration was seen in the embryonating chicken cultures 15 to 48 hours after N.D.V. inoculation. The fibroblasts showed an increase in vacuolization of the cytoplasm and a tendency in the formation of giant cells which contained 3 to 5 nuclei. The only degenerative changes in the adult tissue cultures occurred in normal adult chicken liver and this did not appear until 7 or 8 days after N.D.V. inoculation. There was a gradual degeneration of the liver cells in which the cytoplasm lost its staining properties and broke up into large granules and the cell wall became fragmented. The nucleus, however, was unaltered. Neither the uninoculated cultures nor the cultures inoculated with heat-inactivated N.D.V. showed any degenerative change.

TABLE OF CONTENTS

Page

INTRODUCTION 1

I. The Use of Tissue Culture Techniques in Studying Animal Viruses 3

A. Methods of Demonstrating Virus Activity in Tissue Cultures. 4
1. Animal Infectivity Tests. 4
2. Serological Tests 4
Hemagglutination Test 5
Hemagglutination Inhibition Test. 6
3. Growth Curves Illustrating the Multiplication of Viruses in Tissue Culture. . . 7
4. Abnormal Changes in Tissue Culture Caused by Viruses 9
5. Propagation of Newcastle Disease Virus in Tissue Cultures. 10

II. Choice of Virus 11

A. Description of Newcastle Disease Virus 12
1. Etiology of Newcastle Disease 12
2. Host Range of N.D.V. 14
3. Description of Newcastle Disease in Chickens 15
4. Distribution of N.D.V. in Animal Tissues. . 15
5. Growth of N.D.V. in the Embryonating Egg. . 16
6. Histopathology of Newcastle Disease in Adult Chickens. 16
7. Histological Changes in Embryonating Chickens due to N.D.V. 17

B. The G.B. Strain of Newcastle Disease Virus . . 18

MATERIAL AND METHODS 19

A. Description of Procedure of the Experiment . . 19
1. Choice of Tissue Culture Technique for Virus Propagation 19
2. Preparations for Tissue Cultures. 19
3. Outline of the Experiment 21
4. Description of Tissue Culture Technique . . 23

TABLE OF CONTENTS Continued

Page

B. Methods Used for Determining N.D.V. Activity in Infected Tissue Cultures. 24
1. Infectivity Tests Using Embryonating Chicken Eggs. 24
2. Hemagglutination Tests. 26
3. Methods Used for Animal Immunization. . . . 27

RESULTS. 28

A. Thermolability of the Virus. 28

B. Results of Hemagglutination and Infectivity Tests. 29
1. Hemagglutination Tests with Tissue Cultures Given Heat-inactivated N.D.V. 29
2. Hemagglutination and Infectivity Tests with Embryonating Chicken Tissue Culture. . 30
3. Hemagglutination and Infectivity Tests with Rabbit and Chicken Tissue Culture. . . 32
Hemagglutination Tests. 32
Infectivity Tests 38

C. Changes in the Tissue Cultures Which Were Infected with N.D.V. 40

DISCUSSION 48

SUMMARY. 54

BIBLIOGRAPHY 56

INTRODUCTION

The purpose of this study was to determine whether or not resistance and susceptibility of animals to Newcastle Disease Virus (N.D.V.) could be attributed to specific tissues or organs. The liver, lung and spleen of adult, normal and immunized chickens and rabbits, as well as normal chicken embryo tissue were chosen for tissue cultivation. The chicken represented the naturally susceptible, and the rabbit, the naturally resistant animals to N.D.V. As soon as these tissues attained maximal growth in tissue cultures they were infected with N.D.V. to study the morphological changes and variation in their capacity to support multiplication of the virus.

This was the specific purpose of the work but there was also the more general purpose of seeking any evidence which might contribute to a better understanding of resistance and susceptibility of animal cells to a virus.

Virologists have been limited in many of their practical studies because it has been impossible, in many cases, to detect virus activity by any means other than animal inoculation. Primary isolation of such viruses as those of hog cholera, measles and infectious hepatitis, may be accomplished in only the original host or a few large

animal species. This restricts laboratory investigations greatly due to the high cost and the time involved in maintaining these animals. Most of the recent studies pertaining to the relationship of host cells and viruses have been carried out with the purpose of finding a means of increasing their host range so that small animals may be used. In spite of persistent research, no adequate explanation has been found to account for the resistance of small laboratory animals to these viruses.

On the other hand, there exists a group of viruses which can be propagated readily in small animals as well as in the embryonating chicken egg. Consequently several methods have been developed for determining virus activity. For example, N.D.V., mumps and influenza viruses may be propagated in the embryonating chicken egg; then, the allantoic or amniotic fluids may be used as antigenic suspensions for hemagglutination, hemagglutination-inhibition and complement fixation tests. In addition, N.D.V. may be propagated in most laboratory animals which may be easily maintained for laboratory experimentation, e.g., chickens, hamsters and mice. With these viruses, therefore, the problem involves not an understanding of resistance but an explanation of the factors responsible for the susceptibility of these animals. If these factors could be determined the virologist might in turn find a means of lowering the

resistance of other small animals to the viruses of hog cholera, infectious hepatitis, etc.

I. The Use of Tissue Culture Techniques in Studying Animal Viruses

Viruses were first propagated in tissue cultures when Parker and Nye (1925) carried vaccinia virus through a series of 11 cultures of rabbit testicular tissue. Since that time many of the viruses known to cause disease in animals have been grown in tissue cultures. This technique has been applied to many types of virus studies such as:

(a) effects on the cells which viruses attack

(b) site of virus multiplication

(c) enumeration of conditions essential for virus propagation

(d) details of the mechanisms of natural and acquired immunity caused by viruses

(e) changes in pathogenicity which may occur as a result of cultivation of the virus in tissue cultures especially associated with the possibilities of vaccine production

(f) determination of the effects of various chemical compounds, including antibiotic agents, on the multiplication of viruses

(g) attempts to adapt tissue culture techniques for the purpose of rapid diagnosis of some virus diseases.

A. Methods of Demonstrating Virus Activity in Tissue Cultures

Two methods have been used for demonstrating the multiplication of viruses in tissue culture. The first consists of showing that the fluid withdrawn from a culture exhibits activities characteristic of the virus in question; such as, animal infectivity, hemagglutinating or complement fixing activity. The second method is the demonstration of abnormal cellular changes or an alteration in the pH of the surrounding fluid due to a decrease in tissue metabolism caused by the virus.

1. Animal Infectivity Tests

Animal infectivity tests have proved to be the most reliable for detecting virus multiplication and are necessary to confirm the results of serological and cytological findings. With N.D.V., for example, serial dilutions of the infected tissue culture fluids are inoculated into embryonating chicken eggs and the fifty percent mortality endpoint, LD_{50}, is determined according to the method of Reed and Muench (1938).

2. Serological Tests

Hemagglutination and complement fixation tests provide more rapid means of demonstrating virus activity than do animal infectivity tests. The basic principles of the complement fixation method are the same for viruses as for bacteria. This test may be used with many viruses such as

those of the lympho-granuloma-psittacosis group, and the mumps, influenza-N.D.V. group. The hemagglutination test has the advantage of being more rapid but is limited to a smaller number of viruses, namely the mumps-influenza-N.D.V. group.

Hemagglutination Test: Hirst (1942a) and McClelland and Hare (1941), discovered that influenza viruses agglutinate the red blood cells of fowl. Burnet (1942) reported the agglutination of fowl cells by N.D.V.-infected fluids of the embryonating chicken egg and Levens and Enders (1945) described similar agglutination of sheep red blood cells by mumps virus. The Bureau of Animal Industry (1946) standardized the hemagglutination test which is a modification of the hemagglutination procedure described by Salk (1944). This quantitative measurement has been described in the following manner:

> Two-fold serial dilutions of the virus suspensions were made in physiological saline. To these dilutions a suitable concentration of a red blood cell suspension was added. After thorough shaking the cells were allowed to settle for a period of one to two hours. In the test tubes containing virus the agglutinated red blood cells deposited in a thin film covering the entire portion of the bottom of the tube, while in the tubes containing no detectable virus the cells settled out in the form of a small sharply-outlined button. The endpoint was considered to be the highest dilution of a virus producing maximal hemagglutination and the titer was expressed as the reciprocal of the final dilution of virus. This titer has also been designated as one hemagglutination unit. Thus if one hemagglutination unit were represented by a titer of 1600, two hemagglutinating units would correspond to a titer of 800 and 8 hemagglutinating units a titer of 200.

Two disadvantages in the use of the hemagglutination test for this experiment have been reported. First, when a N.D.V. suspension was treated by heating at 56° C for 40 minutes it was no longer infectious for embryonating chicken eggs but the hemagglutinating activity was only slightly decreased (Durusan, 1949). Thus the hemagglutinating titer was found to be proportional to the sum of the infections as well as the non-infectious virus particles. Secondly, measurement by hemagglutination is restricted to those suspensions in which large quantities of virus are present. An N.D.V. suspension which will infect the embryonating chicken when diluted 1,000,000,000 times will give maximal hemagglutination only up to a dilution of 1600. Gordon (1952) showed that the multiplication of N.D.V. in the embryonating chicken egg increased to a 10^{-6} titer before there was any evidence of hemagglutination activity. The hemagglutinating titer only then showed a proportional increase as the infectivity titer in the embryonating chicken egg increased from 10^{-6} to 10^{-9}.

Hemagglutination Inhibition Test: Hemagglutination has also been widely used for antibody measurement. This adaptation of the test is called hemagglutination inhibition and is based on the principle that the hemagglutinating viruses, when mixed with hemologous immune serum, lose their ability to agglutinate red blood cells. The degree of inhibition gives an estimate of the amount of antibody in

the serum. Two hemagglutination inhibition methods have been used with N.D.V. In the "alpha" procedure, serial dilutions of virus are admixed with a fixed amount of serum, whereas in the "beta" procedure a definite amount of virus suspension containing the hemagglutinating units is admixed with an equal amount of serial serum dilutions before the red blood cells are added. Brandly (1947) reported that there was a 93-percent agreement between the alpha and beta procedures.

3. Growth Curves Illustrating the Multiplication of Viruses in Tissue Culture

Animal infectivity tests and hemagglutination tests have been used to prepare growth curves illustrating the multiplication of viruses in tissue cultures. Of particular interest are the growth curves established for poliomyelitis virus by Salk (1952) and Syverton (1952), as well as those for influenza and mumps viruses set up by Enders (1950). Enders established growth curves for influenza and mumps viruses in embryonating chicken tissue cultures using the hemagglutination technique, whereas Salk and Syverton made their determination for poliomyelitis virus in monkey testicular tissue cultures using monkey infectivity tests.

Growth of these viruses occurred in a discrete cycle characterized by:

(a) a lag phase - in which the virus required time for infection and multiplication in the cell

(b) a phase of rapid virus multiplication which reflects the release of virus from within infected cells to the extracellular fluid

(c) a plateau phase which represented a decrease in virus production

(d) a stage in which the virus production was reduced to zero.

Influenza virus showed a very short lag phase, then increased rapidly attaining a peak hemagglutination titer at 48 to 72 hours. Thereafter relatively little additional virus was produced. Mumps virus increased relatively slowly usually reaching maximum concentration in the fluid phase on the twelfth day of virus cultivation. There was no visual evidence of cytological changes in the tissue cultures in which either mumps or influenza virus were propagated.

The duration of the growth cycle of poliomyelitis virus was approximately 10 days. Significant amounts of virus were not released into the liquid phase until the fifth to seventh day. Occasionally, two successive cycles of poliomyelitis virus production were observed in a single culture. This was shown by a secondary rise in the quantity of the virus released into the liquid from the eighth to the eighteenth day of cultivation. By 30 days virus was not detectable either in the tissue or liquid phase. The first cycle was correlated with the destruction of cells which

extended from explants while the second cycle occurred only if there were a sufficient number of surviving host cells within the explants.

4. Abnormal Changes in Tissue Culture Caused by Viruses

Viruses have been shown to cause degenerative changes in tissue culture cells as well as the formation of characteristic intracellular inclusion bodies. Huang (1943) showed that the virus of equine encephalomyelitis completely destroyed embryonating chicken cells within 72 hours. Salk (1952) described a slower type of degeneration which was produced in the fibrocytes of monkey testicular tissue infected with poliomyelitis virus. First, there occurred various granular changes and vacuolization in the cytoplasm, then a varying degree of cytolysis and a decrease in the development of new fibrocytes surrounding the tissue explants took place. Most of the nuclei were shrunken and pyknotic, but a few appeared swollen and were stained less intensely. Bland and Canti (1935), using the tissue culture technique, found that the elementary bodies of psittacosis virus pass through a definite cycle of development and that the plaque or inclusion body is essentially a colony of elementary bodies.

The effect of a virus on tissue metabolism may be indicated by a decreased acid production in the tissue culture. Huang (1943) noted that chicken embryonating tissues from 48-hour cultures of equine encephalomyelitis virus were no longer capable of reducing methylene blue.

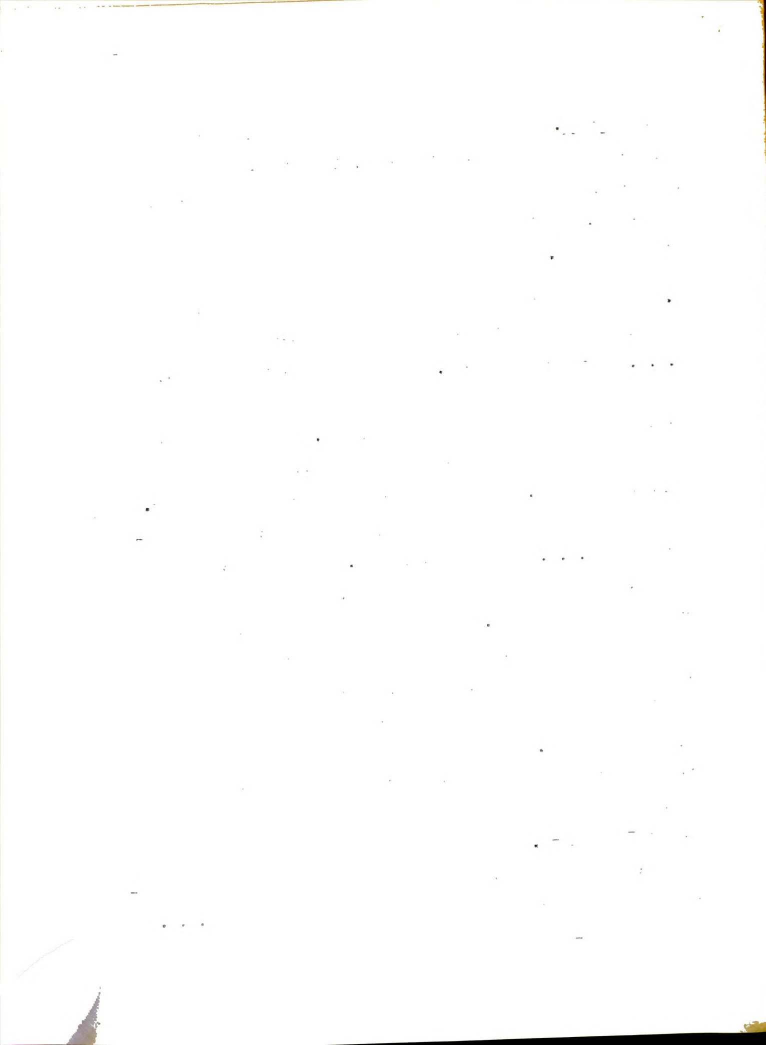
Enders *et al.* (1949) found that acid production in flask cultures of human embryonic brain, intestine, and skin-muscle inoculated with poliomyelitis virus after variable intervals, declined more rapidly than in control cultures without virus.

5. Propagation of Newcastle Disease Virus in Tissue Cultures

Topaccio (1934) reported the first attempt to propagate N.D.V. in tissue cultures. Embryonating chicken tissue cultures were inoculated with a virus suspension prepared from the spleen of an infected chicken. Subcultures were made every three to five days and 31 successive passages were carried out. Virus was recovered from all passages.

Bankowski and Boynton (1948) also reported the propagation of N.D.V. in tissue cultures. Embryonic chicken livers, infected 36 hours previously, were cultured by the suspended cell method. After each tissue culture passage portions of the suspended cells were used for embryonating chicken egg inoculation, so that every other passage was an egg passage and not an uninterrupted series of tissue culture passages. Subcultures were made every 72 hours from the tissue culture passage to the embryonating egg and at this time the LD_{50} of the infected tissue culture ranged from 10^{-4} to 10^{-7}.

Scott (1954) described a simplified tissue culture procedure for the propagation of the Roakin strain of N.D.V. in the chorio-allantoic membrance of the embryonating



chicken. Growth curves were established using infectivity tests as well as hemagglutination tests. It was found that a peak LD_{50} titer of 10^{-8} appeared in 24 hours and a peak hemagglutination titer of 128 appeared in 36 hours.

II. Choice of Virus

Newcastle Disease Virus (N.D.V.) was chosen in this experiment for the following reasons. First, N.D.V. activity may be demonstrated by several laboratory procedures: by infecting the embryonating chicken egg, by the complement fixation and the hemagglutination tests. Secondly, this virus is relatively well known because of its relationship to the mumps and influenza group of viruses. A large part of recent investigations on host-cell-virus relationships have been carried out with the mumps-influenza-N.D.V. group of viruses, and, therefore, any additional findings may be more easily fitted into an explanation of the mechanisms of resistance and susceptibility. A third reason for choosing N.D.V. is the fact that it is a relatively thermolabile virus. Durusan (1949) found that N.D.V.-infected allantoic fluids were no longer infectious for the embryonating chicken egg after an incubation period of five days at 37° C. This is a significant factor in tissue culture studies because if the virus is not decreased by a temperature of 37° C (the temperature of incubation) it is difficult to prove that virus multiplication, not passive

transfer of the virus, has occurred. It follows, therefore, that, if the fluids of the N.D.V.-infected cultures still prove infectious after being incubated five days at 37° C, multiplication of the virus must have taken place.

A. Description of Newcastle Disease Virus

1. Etiology of Newcastle Disease (synonym: avian pneumoencephalitis)

Newcastle disease is an epornitic infection of poultry characterized by a viremia and signs of involvement of the respiratory, central nervous system, and gastro-intestinal organs. Outbreaks of Newcastle disease due to immunologically identical strains have occurred in many parts of the world; for example, in England, U.S.A., Asia, Africa and Australia. Newcastle disease primarily affects chickens and turkeys.

The etiological agent of Newcastle disease is a virus of medium size. Burnet and Ferry (1933) showed that the particle size of N.D.V. was 80-120 mu. The virus particle is filamentous (Bang, 1946) or spermlike (Cunha, _et al_., 1947). It is filterable through Berkefeld V. N and W candles, Chamberland L_3 and L_5 filters, and Seitz pads (ST-3). The virus may be preserved in 50 percent glycerine, in the frozen state, or by lyophilization (Burnet and Ferry, 1934). Inactivation of the virus has been accomplished by ultraviolet light when a clear suspension was irradiated by

1600-1800 A for 0.8-1.08 seconds. It may also be inactivated by a 1/5000 dilution of formalin and by N/50 sodium hydroxide in one hour (Brandly, et al., 1946a).

The maximal stability of N.D.V. lies between pH 4 to pH 11 (Brandly, Moses and Jones, 1946d). Miller and Stanley (1944) found that the pH of the medium had only a minor effect on the hemagglutinating activity of the virus in a range of pH 6 to pH 8.

Burnet (1945) found that, if the allanto-amniotic fluid suspension of N.D.V. was held at 65° C for 15 minutes, there was complete loss of infectivity but the hemagglutinating activity was still 50 percent of the original titer. Durusan (1949) also found that the hemagglutinating activity of the N.D.V.-infected allantoic fluid was more thermostable than the infectivity of the virus. The initial LD_{50} of the virus was $10^{-9.32}$ and its hemagglutinating titer was 1280. When the virus was stored for 13 weeks at 4° C the infectivity titer decreased to $10^{-1.62}$ whereas the hemagglutinating titer remained constant. When held at room temperature (22°-27° C) for 30 days the infectivity decreased to less than 10^{0} but the hemagglutinating titer decreased only from 1280 to 640. Durusan also found that when the virus suspension was held at 62° C there was complete loss of infectivity after 15 minutes but hemagglutinating activity was not lost until after 25 minutes.

The correlation of this study and Durusan's experiment on the thermolability of N.D.V. at 37° C has already been mentioned. He found that when the virus suspension was incubated at 37° C the initial LD_{50} of $10^{-9.32}$ dropped to $10^{-5.5}$ after one day, to $10^{-3.62}$ after two days, to less than 10^{-3} after three days and to $10^{0.21}$ after four days. During the same period, however, the hemagglutination titer changed only from 1280 to 640 and remained at 640 for more than eight days. For this reason it is evident that the hemagglutination test is of value only when an increase in hemagglutination titer occurs.

2. Host Range of N.D.V.

Newcastle disease occurs naturally in chickens, turkeys, pheasants, guinea fowl, sparrows, crows, francolines and parrots (Beaudette, 1942). Experimental infection has been achieved in chick embryos, ducks, geese, pigeons and several varieties of wild birds. Mice, hamsters, cotton rats and monkeys may be infected experimentally with N.D.V. resulting in a meningo encephalitis. Over 300 serial passages in hamsters and 30 serial passages in mice have been carried out (Reagan, 1952a, b). Several accidental infections have occurred in human beings handling infected fowl or working with the virus (Burnet, 1943; Hungherr, _et al._, 1949).

Rabbits, cows, horses, pigs, sheep, ferrets and guinea pigs are considered to be resistant to N.D.V. (Beaudette,

1943). A meningo-encephalitis may occur in rabbits after intracerebral inoculation but the virus has not been transferred from rabbit to rabbit.

3. Description of Newcastle Disease in Chickens

The present opinion is that the pneumonic and central nervous system (CNS) symptoms deominate the American type of Newcastle disease, while respiratory and gastro-intestinal symptoms are most prevalent in the Asian and European type (Brandly, et al., 1946a). In naturally-infected chickens the incubation period is 4 to 11 days and is followed by characteristic symptoms of pneumonia or central nervous system involvement. Death may ensue after two to four days.

4. Distribution of N.D.V. in Animal Tissues

Karzon and Bang (1950) and Hofstad (1951) reported the following results from quantitative studies of the American type of N.D.V. in tissues of experimentally-infected chickens. When the virus was inoculated by the intranasal, intravenous or intramuscular routes it was recovered from the blood, spleen, kidneys, lung, brain, and trachial and intestinal contents within 24 hours. Virus concentrations as high as 10^{-5} and 10^{-7} (embryo lethal doses) were found by the fourth day then gradually decreased until the virus could no longer be isolated after the seventh day. Neutralizing antibodies against N.D.V. and hemagglutinin-inhibiting antibodies were demonstrated five to seven days after N.D.V. inoculation;

thus, the virus disappeared as soon as the antibody concentration in the blood became relatively high. The virus neutralization and the hemagglutination-inhibition tests yielded parallel results early in the convalescent period but later (22 to 100 days) the hemagglutinin-inhibiting activity of the serum showed a steep decrease.

Newcastle disease virus has been isolated from the liver of infected chickens but never in sufficiently high concentrations to yield consistant titers.

5. Growth of N.D.V. in the Embryonating Egg

When N.D.V. was inoculated into the allantoic cavity of the embryonating chicken egg it was rapidly adsorbed by the cells of the allantoic membrane, (Gordon, 1952). Within the first hour the LD_{50} titer decreased from $10^{-4.5}$ to $10^{-1.6}$ and the hemagglutinating activity titer was lost. The infectivity titer increased when tested every four hours, until it reached LD_{50} titers of 10^{-6} at 12 hours and $10^{-8.3}$ at 16 hours. The hemagglutinating activity did return after 12 hours; then, the titer increased from 10 to 1280 by the 24th hour. Meanwhile, the infectivity titer showed no increase in the 16 to 48 hour period.

6. Histopathology of Newcastle Disease in Adult Chickens

Grossly the disease in fowl produced multiple focal necrosis in the viscera, and hemorrhages, especially in the respiratory and alimentary tracts; at times, an interstitial pneumonitis was observed (Brandly, _et al._, 1946a, b).

Cytologically, the exudative lesions in the lung consisted of cellular and serous accumulations in the peribronchial alveoli with extension into the lumen of the regional tertiary branches. The exudate was composed of various types of desquamated pulmonary cells including swollen, vacuolated (foam) cells, and disintegrating heterophiles and erythrocytes. The central nervous system showed perivascular infiltration, a honey-combed appearance of degenerated neurons and gliosis near the altered neurons in the lumbar cord, medulla and cerebellar nuclei (Brandly, et al., 1946b). In addition to focal necrosis in the liver, pericarditis, and secondary inflammatory changes in the abdominal air sacs Fukushima (1932) found hyperplasia of the intestinal lymphoid patches with occasional necrosis and hyaline degeneration of the splenic follicles with development of network-like homogeneous masses.

7. Histological Changes in Embryonating Chickens due to N.D.V.

Cytological changes in embryonating chicken eggs due to N.D.V. were first described by Burnet (1942). When the virus was inoculated into the amniotic sac, multiple chorioallantoic hemorrhages, hemorrhagic encephalitis and respiratory lesions appeared within 36 hours. In sections of the lung cellular changes were seen to be widespread. The earliest change recognizable was a distortion of the nucleus of epithelial cells giving a clear oval area within which

the chromatin was piled up into masses of various shapes and distribution. In tracheal smears large vacuolated cells were found arranged in agglutinated masses in which it was impossible to pick out the outlines of individual cells. Some cells showed a nucleus broken into many heavily stained circular fragments as well as a great degree of cytoplasmic degeneration.

B. The G.B. Strain of Newcastle Disease Virus

The G.B. strain of N.D.V. was chosen for this work because it is a well-known field and laboratory strain and it is highly pathogenic for chickens. This strain was isolated in May, 1948 by Boney and Grumbles from an outbreak of a severe nervous and respiratory infection of chickens (Boney, 1948). Chickens infected experimentally with the virus show different symptoms depending upon the route of inoculation. Those exposed by intramuscular injection develop a profuse diarrhea, weakness, and general paralysis with tonoclonic symptoms. The same symptoms occur in chickens exposed by the intranasal route but there is usually an interstitial pneumonia as well. Morbidity and mortality up to 100 percent may occur in young susceptible chickens. The LD_{50} for lo-day old embryonating chicken eggs is 10^{-9}.

MATERIAL AND METHODS

A. Description of Procedure of the Experiment

1. Choice of Tissue Culture Technique for Virus Propagation

The simplest procedure which could be readily adapted to large-scale tissue cultivation was chosen. The tissues were minced, then embedded in a plasma matrix in the bottom of a Petri dish (9 cm x 1.5 cm). When the tissues became fixed in the plasma they were flooded with Hank's nutrient solution. In this way it was possible to prepare and maintain several hundred Petri dish cultures at one time.

This method provided an additional advantage in that representative cultures could be examined microscopically at a magnification of 100x to 970x. This was accomplished by simply removing the nutrient fluid, covering the tissue explant with a glass cover slip and lowering the objective of the microscope into position directly over the tissue growth. For comparative studies and photomicrographs permanent preparations were made of the Petri dish cultures by staining the tissue with Giemsa stain.

2. Preparations for Tissue Cultures

Heparinized plasma was used for the mat in which the tissues were embedded. The plasma was filtered through Seitz filter (St-3), heated in a water bath at 56° C for

30 minutes and tested for bacterial sterility as well as hemagglutinating-inhibiting properties. All the plasma used showed a hemagglutinating-inhibition titer of less than 1/40 which according to the standards of the Bureau of Animal Husbandry is considered to be within the normal range. A quantity of 0.5 to 1.0 ml of plasma was sufficient to cover the bottom of a Petri dish.

Hank's nutrient medium, a balanced physiological solution, recommended by Enders (1950) as a tissue culture nutrient, was used for all cultures. Ten milliliters of the nutrient solution was sufficient to cover each Petri dish culture and to sustain growth of the cells for a period of four to seven days. Phenol red, an indicator with a color range between pH 6.8-8.4 was incorporated in this solution. The hydrogen ion concentration of the nutrient was adjusted to a pH 7.2-7.4 before it was added to the cultures. The G.B. strain of N.D.V. was used for both tissue culture inoculation and animal immunization. The cultures were inoculated by replacing the fluids with the N.D.V.-infected allantoic fluid, diluted 1/200 in Hank's nutrient solution. This dilution represented eight hemagglutinating N.D.V. units. The succeeding fluid changes were made with Hank's nutrient solution alone.

In the first two experiments all fluids were checked for bacterial sterility using blood plates and N.I.H. thioglycollate sterility-broth, but this was discontinued when

it was found that the Petri dish tissue cultures provided a more sensitive test for bacterial growth. Often the broth and blood plates failed to show bacterial contamination while the stained Petri dish cultures showed a few bacteria.

Bacterial and mycotic contamination was reduced to less than five percent by incorporating 100 units of penicillin, 500 micrograms of streptomycin and two Candida albicans units of endomycin* in each milliliter of Hank's nutrient solution. Endomycin, a new antibiotic, was first prepared by Gottlieb (1951). It inhibits the growth of a wide variety of fungi pathogenic to plants and animals, many gram positive and a few gram negative bacteria.

Chemically clean glassware was obtained by washing with the detergent Tetra D and then rinsing twice in tap water and twice in distilled water.

3. Outline of the Experiment

Ten to twenty Petri dish cultures were prepared for each "tissue culture group" in order to provide a sufficiently large number of representative cultures for each virus inoculum (Table I). The term "tissue culture group" was coined for convenience to designate five to ten Petri dish cultures of one type of tissue embedded in either rabbit or chicken plasma. Each group consisted of tissues

---

*The author is grateful to the Upjohn Company for supplying the endomycin utilized in this experiment.

FIGURE I

OUTLINE OF THE EXPERIMENT

The following is an outline of one complete experiment which comprised 27 "tissue culture groups". Each tissue culture group consisted of all the cultures prepared from one type of tissue embedded in either chicken plasma or in rabbit plasma, for example, group 1 represented all the embryonating chicken tissues embedded in chicken plasma, which included tissue receiving (a) heat-inactivated N.D.V., (b) active N.D.V., and (c) control cultures which were Hank's nutrient solution instead of N.D.V. solution.

A. Cultures of tissues from embryonating chicken egg
1. Embryonating chicken tissue embedded in normal chicken plasma
2. Embryonating chicken tissue embedded in normal rabbit plasma

B. Cultures of tissues from normal adult chickens
3. Lung tissue embedded in normal chicken plasma
4. Lung tissue embedded in normal rabbit plasma
5. Liver tissue embedded in normal chicken plasma
6. Liver tissue embedded in normal rabbit plasma
7. Spleen tissue embedded in normal chicken plasma
8. Spleen tissue embedded in normal rabbit plasma

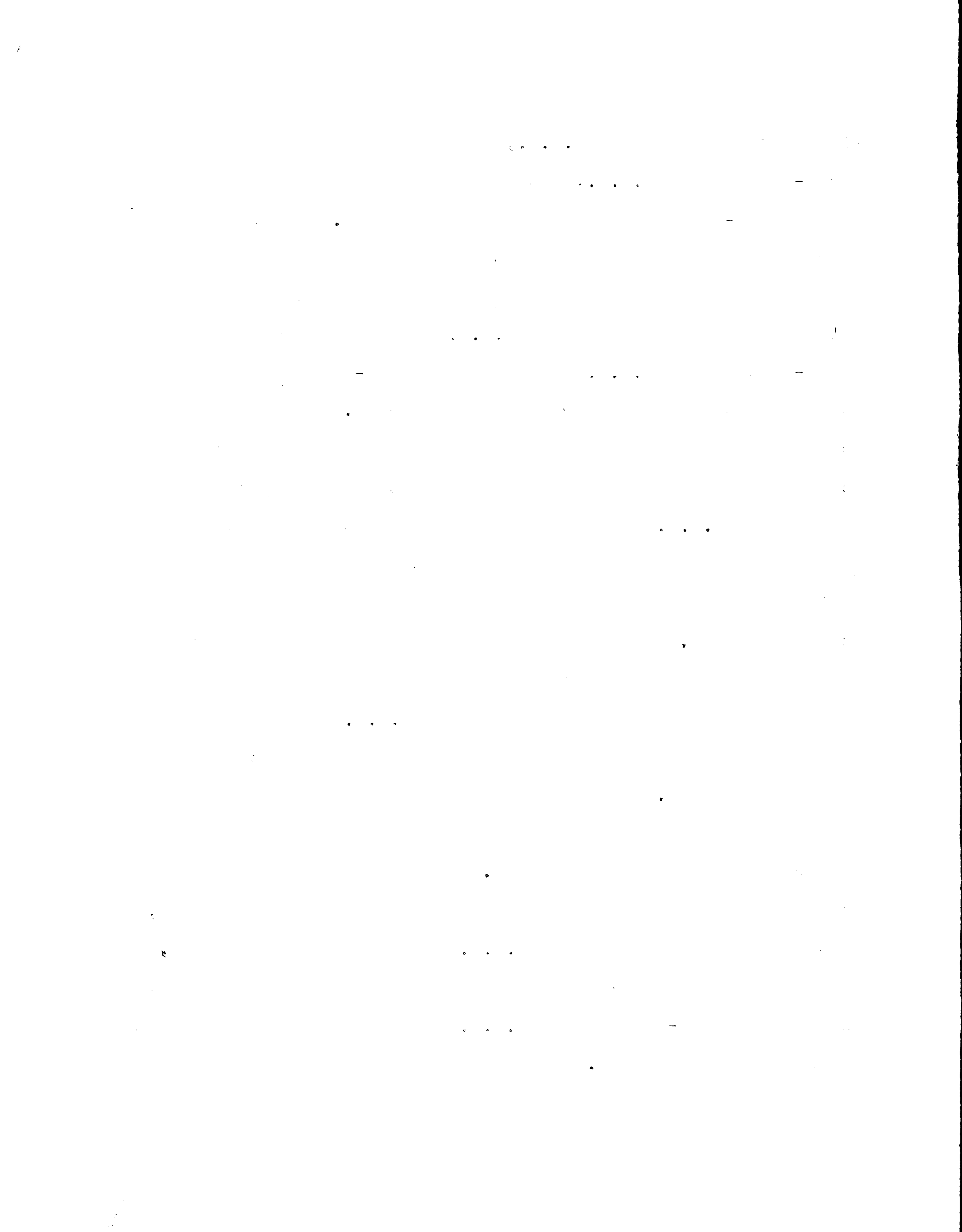
C. Cultures of tissues from immune adult chickens
9. Lung tissue embedded in normal chicken plasma
10. Lung tissue embedded in normal rabbit plasma
11. Liver tissue embedded in normal chicken plasma
12. Liver tissue embedded in normal rabbit plasma
13. Spleen tissue embedded in normal chicken plasma
14. Spleen tissue embedded in normal rabbit plasma

D. Cultures of tissues from normal adult rabbits
15. Lung tissue embedded in normal chicken plasma
16. Lung tissue embedded in normal rabbit plasma
17. Liver tissue embedded in normal chicken plasma
18. Liver tissue embedded in normal rabbit plasma
19. Spleen tissue embedded in normal chicken plasma
20. Spleen tissue embedded in normal rabbit plasma

E. Cultures of tissues from immune adult rabbits
21. Lung tissue embedded in normal chicken plasma
22. Lung tissue embedded in normal rabbit plasma
23. Liver tissue embedded in normal chicken plasma
24. Liver tissue embedded in normal rabbit plasma
25. Spleen tissue embedded in normal chicken plasma
26. Spleen tissue embedded in normal rabbit plasma
27. Lymph node tissue embedded in normal rabbit plasma

inoculated with active N.D.V., plus tissues inoculated with heat-inactivated N.D.V., plus five to ten Petri dish cultures of the non-infected tissue used for controls. Thus, the first tissue culture group listed in Table I included the cultures of normal adult chicken lung embedded in chicken plasma which received active N.D.V. and those which received heat-inactivated N.D.V. as well as the non-infected lung tissue cultures which were used as controls. Five to ten cultures were prepared for each tissue so that representative cultures could be withdrawn at different intervals, to be tested for N.D.V. activity and the tissue stained by the Giemsa method for microscopic examination and enough cultures would be left for observation until the end of the experiment. The tissues were embedded in both rabbit and chicken plasma to determine if either plasma would enhance or suppress multiplication of N.D.V., or if the ability to support growth of the virus was a property of the tissue alone.

An equal number of tissue cultures was maintained as controls during the same period. These cultures were subjected to the same conditions, and the same fluid changes, with the exception that the N.D.V. suspension was not added. In one experiment an additional set of controls was included, in which a heat-inactivated N.D.V. suspension was added to each type of tissue culture. This virus suspension was incubated for seven days at 37° C so that it was no longer infectious



for the embryonating chicken egg but retained a hemagglutinating titer of 1/800.

4. Description of Tissue Culture Technique

The adult tissues used consisted of lung, liver and spleen from normal, as well as N.D.V.-immune rabbits and chickens. Lymph node tissue from normal and immune rabbits was also cultured. Embryonic tissue, consisting of chorio-allantoic membrane, brain and torso of 11-day embryonating chicken eggs were used for all preliminary studies because they were most easily obtained and gave consistently uniform growth of fibrocytes and round cells.

Fragments of the tissues from animals were minced within one hour after removal. Each tissue was placed in a Petri dish and flooded with warm nutrient solution, washed free of extraneous blood and tissue fluids and then transferred to a fresh nutrient solution where it was cut with a fine, curved scissors. The average fragment size approximated 0.1 mm in diameter.

These small fragments were embedded in the thin layer of normal chicken or rabbit plasma covering the bottom of a Petri dish and allowed to become fixed before Hank's nutrient solution was added. Then the tissue cultures were incubated for four days at 37° C. The nutrient fluid was not changed during this period unless the medium became acid. This occurred usually with embryonic and splenic

tissue. The four-day incubation period was sufficient to assure the growth of even the most slowly developing adult tissues so that all cultures could be maintained simultaneously, be given the same virus inoculum, and be subjected to the same experimental conditions.

On the fourth day the nutrient fluid was withdrawn asceptically using separate pipettes for each culture to avoid cross infection. The cultures were then flooded with Hank's nutrient solution containing eight hemagglutinating units of N.D.V. On the fourth, eighth, twelfth, and eighteenth days after virus infection, the nutrient solution was removed and replaced with fresh Hank's nutrient fluid. Samples of the fluid from each culture were withdrawn at intervals of 2, 6, 12, 24, 36, 48, 72 and 96 hours after the N.D.V.-infected suspension was added, as well as just before and two hours after each subsequent fluid change. Samples from each type of tissue culture were pooled for each incubation period and stored at -20° C until they could be tested for virus activity. Petri dish cultures representing similar incubation periods were stained by the Giemsa method for microscopic examination.

B. Methods Used for Determining N.D.V. Activity in Infected Tissue Cultures

1. Infectivity Tests Using Embryonating Chicken Eggs

All samples of the tissue culture fluids were tested for N.D.V. activity by inoculating 0.2 ml of the fluid into

the allantoic cavity of 10-day embryonating chicken eggs. Infectivity tests were also carried out on suspensions of each type of tissue which were prepared at the time they were removed from the animals.

If N.D.V. was present in the tissue culture fluids or in the suspensions of the animal tissues, death of the embryo occurred within two to five days. At the time of death the eggs were placed in the refrigerator (+4° C) for six hours and then the allantoic fluids were withdrawn and tested for bacterial sterility as well as hemagglutinating activity.

Quantitative infectivity tests were carried out with the samples of tissue culture fluids which showed positive infectivity in order to determine the 50 percent mortality endpoint (LD_{50}). Samples from culture fluids of embryonating chicken tissue were tested at intervals of 2, 6, 12, 24, 48, 72 and 96 hours after N.D.V. inoculation to determine the titer of virus. The LD_{50} titers of the fluids removed from rabbit and chicken tissue cultures 96 hours after virus inoculation were also determined.

The eggs which were used were received from W. S. Hannah and Sons of Grand Rapids, Michigan. These eggs were collected from flocks which had not received N.D.V. vaccine.

In order to confirm the work of Durusan (1949), concerning the thermolability of N.D.V. at +37° C, infectivity tests were carried out with samples of an undiluted

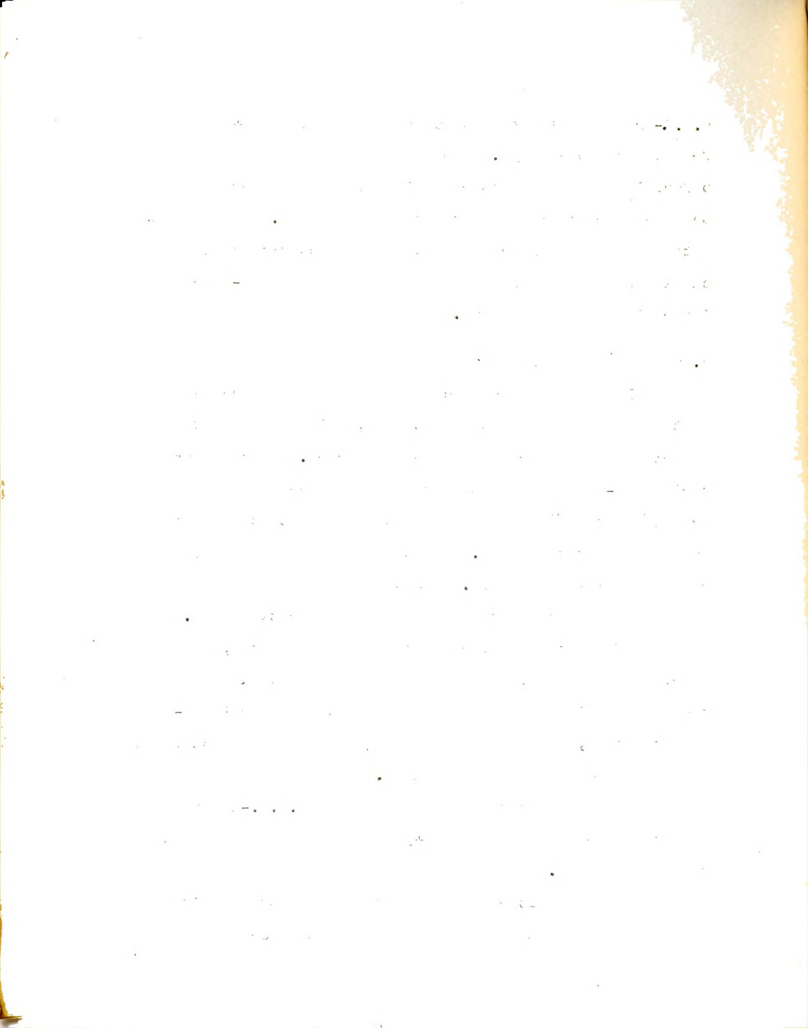
N.D.V.-infected allantoic suspension incubated at +37° C from one to seven days. Serial dilutions were also set up before incubation to determine if the diluted virus would be more thermolabile than the undiluted virus. Hemagglutination tests as well as quantitative infectivity tests were carried out with samples of these solutions at 24-hour intervals up to seven days.

2. Hemagglutination Tests

Pooled samples from each type of tissue culture were tested for hemagglutinating activity, using the procedure described by the Bureau of Animal Industry. In this method serial two-fold dilutions of the virus suspension ranging from 1/100 to 1/6400 were prepared and to each test tube an equal quantity of a 0.25 percent suspension of chicken red blood cells was added. The test tubes were shaken and allowed to stand at room temperature for two hours. Results of the test were recorded after 30 minutes, one hour, and two hours. The endpoint was considered to be the highest dilution of the virus producing maximal hemagglutination, and the titer was expressed as the reciprocal of the final dilution of the virus.

The hemagglutinating activity of the N.D.V.-infected allantoic fluids incubated at +37° C from one to ten days were also tested.

In order to determine if the tissues of the rabbit and chicken possessed either hemagglutinating or



hemagglutination-inhibiting activity before they were used for tissue cultures, ten percent suspensions of each type of tissue were prepared, centrifuged and the supernatant fluids were withdrawn for these tests. In the hemagglutination-inhibition test a modified B-procedure was used, two units of N.D.V. suspension admixed with serial two-fold dilutions of the tissue fluids ranging from the undiluted fluid to a 1/64 dilution.

3. Methods Used for Animal Immunization

Young adult rabbits and chickens were given four 0.5 milliliter doses of N.D.V.-infected allantoic fluid intranasally at weekly intervals. For the first and second inoculations, heat-inactivated virus was given. Active N.D.V.-infected allantoic fluid was then used for the third and fourth inoculations as well as for the challenging dose which was given three weeks later. Three months after the first inoculation the animals were bled and their sera were tested for hemagglutinin-inhibiting activity. Those animals showing a titer of at least 1/320 were killed and their tissues removed for tissue cultivation. In this case the standard Beta procedure for the hemagglutination-inhibition test was used. A suspension containing 10 units of N.D.V. was mixed with an equal quantity of serial two-fold dilutions of serum, and incubated at room temperature for 10 minutes before adding 0.25 percent suspension of chicken red blood cells.

RESULTS

A. Thermolability of the Virus

The undiluted solution of the G.B. strain of N.D.V. showed a similar thermolability when incubated at $+37°$ C as did the strain of N.D.V. used by Durusan. All of the G.B. solutions which were diluted beyond 10^{-1} showed a sharper decrease in the LD_{50} titers within four days. After two days the LD_{50} titers of the diluted solutions decreased to 10^{-4}, after three days to 10^{-1} and after four days the virus was completely inactivated. The hemagglutination titers decreased from 1600 to 800 during the first 24 hours, then remained at this titer for the seven-day incubation period. Since the virus inoculum for the tissue cultures consisted of a 1/200 solution these hemagglutination titers would correspond to 16 and 8 respectively. It follows, therefore, that if virus was recovered from the tissue culture solution which showed an infectivity titer higher than 10^{0} or a hemagglutination titer higher than 8, multiplication of the virus had taken place in the tissues.

Similarly, if the hemagglutination titer of the undiluted N.D.V.-infected allantoic fluid was 1600, this fluid, when diluted to 1/200 to contain eight hemagglutinating units represented a hemagglutinating titer of eight.

For example, the N.D.V. inoculum containing eight hemagglutinating units could be diluted serially 1/2, 1/4, 1/8 and still produce maximal hemagglutination. It follows, then, that when a titer higher than eight could be recovered from a tissue culture fluid, that tissue had supported growth of the virus.

B. Results of Hemagglutination and Infectivity Tests

Infectivity and hemagglutination tests were negative on all the control tissues and tissue cultures, i.e., in samples of all the tissue removed from the animal and in all cultures of these tissues which were not given the N.D.V. inoculation. Infectivity tests were negative also on all tissues which received heat-inactivated N.D.V. None of the tissue suspensions from the immune chicken or rabbit showed hemagglutination-inhibiting activity.

1. Hemagglutination Tests with Tissue Cultures Given Heat-Inactivated N.D.V.

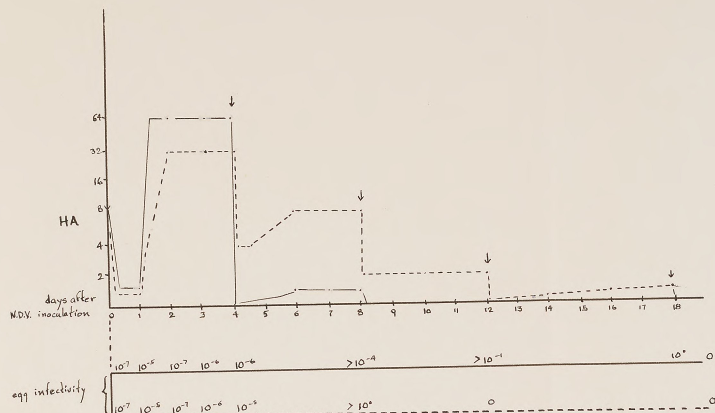
When the heat-inactivated N.D.V. suspension was added to the tissue cultures the initial hemagglutination titer remained constant in all the cultures with the exception of those of the lung and liver of the immune chicken and the tissues cultured from the immune rabbit. The titer of the fluids from the lung and liver of the immune chicken and the lung and spleen of the immune rabbit dropped to a titer of four within two hours, and remained constant

during the next 94 hours. The titer of the fluids removed from the liver cultures of the immune rabbit also decreased to four then to zero within 24 hours. None of the fluids withdrawn from any of the tissue cultures after the first nutrient change showed any hemagglutination activity (Figs. II-VI).

2. Hemagglutination and Infectivity Tests with Embryonating Chicken Tissue Culture

Embryonating chicken tissue cultures were used for all the preliminary tests to determine the growth curve of N.D.V. in tissue cultures (Fig. II). Within two hours after virus inoculation the fluids from these tissue cultures showed a rapid decrease of hemagglutinating activity from eight to two due to adsorption of the virus by the tissue. Within six hours hemagglutination occurred only in the undiluted fluids and did not increase in titer until after 24 hours. Fluids from embryonating chicken tissues which were embedded in chicken plasma thereafter showed a rapid increase in hemagglutination titer reaching a peak titer of 64 in 36 hours. This titer was constant from 36 to 96 hours, then disappeared for two to six hours after the first fluid change. After eight days hemagglutinating activity appeared in the undiluted fluids but was lost completely after the second fluid change.

In the fluids from the embryonating chicken tissues which were embedded in rabbit plasma the hemagglutinating

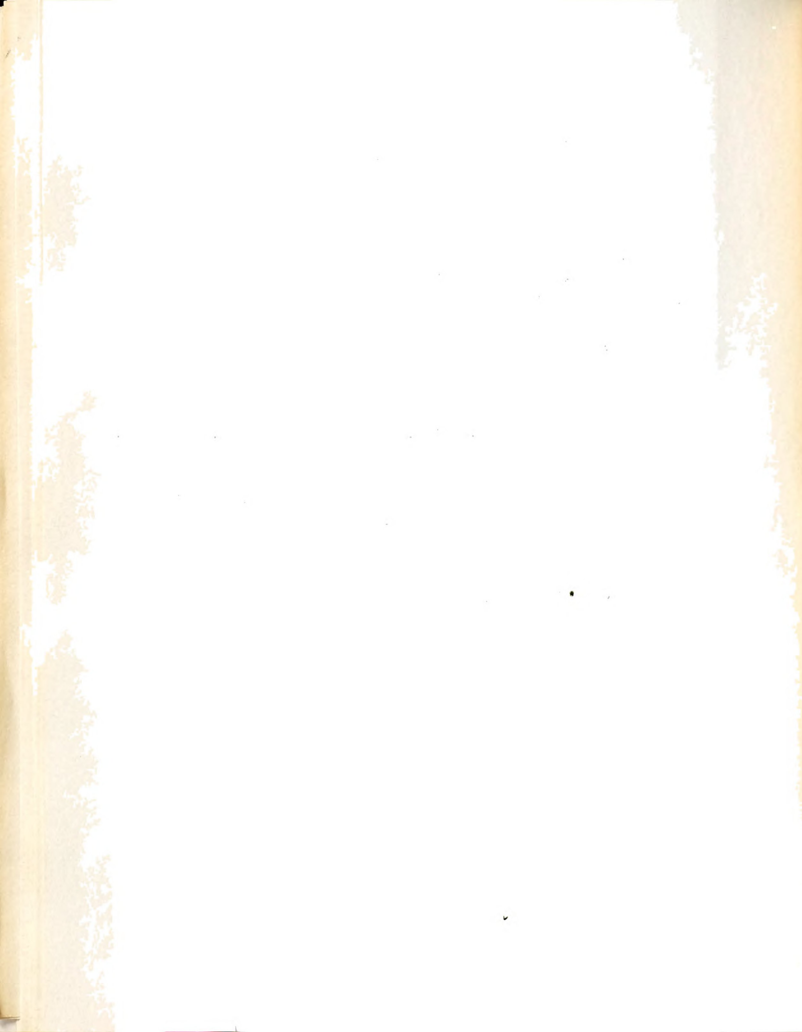


HEMAGGLUTINATION AND INFECTIVITY TITERS OF N. D. V. IN EMBRYONATING CHICKEN TISSUE CULTURES EMBEDDED IN CHICKEN AND IN RABBIT PLASMA

FIG. II

———— N. D. V. TITERS IN CULTURES EMBEDDED IN CHICKEN PLASMA

-------- N. D. V. TITERS IN CULTURES EMBEDDED IN RABBIT PLASMA



activity increased more slowly reaching a peak titer of 32 within 48 hours. After the first fluid change this titer decreased from 32 to four but then rose again to eight on the sixth day and remained at this titer until the eighth day. After the third fluid change hemagglutinating activity could still be recovered from the untiluted fluids of the embryonating chicken tissues which had been cultured in a rabbit plasma matrix.

Fluids were withdrawn from these tissue cultures at intervals of 2, 24, 48, 72 and 96 hours after N.D.V. inoculation for infectivity and hemagglutination tests. Since the virus inoculum consisted of a 1/200 solution the LD_{50} of the initial solution was 10^{-7}. This titer dropped to $10^{-5.82}$ within 24 hours but rose to an LD_{50} of 10^{-7} within 36 hours, then decreased to $10^{-6.39}$ after 72 and 96 hours. Fluids from the embryonating chicken tissue embedded in chicken plasma showed infectivity up to eight days while those embedded in rabbit plasma were infectious for 12 days.

3. Hemagglutination and Infectivity Tests with Rabbit and Chicken Tissue Culture

There was little difference between the hemagglutination titers of the tissues which were embedded in a rabbit plasma and in chicken plasma matrix (Figs. III-VI).

Hemagglutination Tests: When active N.D.V. was added to the tissue cultures of the rabbit and chicken their hemagglutination titer decreased from eight to four or one

within two to six hours (Figs. III-VI). This was followed by a period of rapid virus production in which the titers rose to 32 or 64 within 36 to 96 hours in all the tissue cultures except those of the liver (Figs. IIIb, IVb) and spleen (Figs. IIIc, IVc) of the immune chicken and in the tissues from the immune rabbit (Figs. V, VI). Fluids from tissue cultures of spleen from the immune chicken showed a hemagglutination titer or 16 within 48 hours but did not rise above this titer (Fig. IIIc, IVc). The most remarkable loss of hemagglutinating activity was found in the fluids removed from the immune chicken liver cultures (Figs. IIIb, IVb) and in all the tissues cultured of the immune rabbit (Figs. V, VI). In the period within two to 96 hours following N.D.V. inoculation, hemagglutinating activity was found only in the undiluted fluids from the immune chicken liver cultures, and disappeared after the first fluid change. In the immune rabbit tissue cultures the hemagglutination titer decreased to one after two hours but disappeared completely within 24 hours.

It was found that the greatest variation in hemagglutinating activity in the tissue cultures occurred during the period after the first fluid change, i.e., from four to eight days after N.D.V. inoculation (Figs. III-VII). By the end of this period all the normal rabbit and chicken tissue cultures, with the exception of the normal chicken lung, showed a hemagglutination titer of four. In the normal chicken lung the hemagglutination titer increased

from four to 16 in the cultures embedded in chicken plasma (Fig. IIIa), and from four to eight in the cultures embedded in rabbit plasma (Fig. IVa). In the immune chicken lung cultures there was also a four-fold increase in the hemagglutination titers. The immune chicken spleen cultures showed hemagglutinating activity only in the undiluted fluids (Figs. IIIc, IVc).

After the second and third fluid changes hemagglutinating activity could not be found in the normal tissues of the rabbit and chicken but reappeared within 48 to 96 hours in the undiluted fluids. Hemagglutinating activity disappeared in most of the tissue cultures after the fourth fluid change, i.e., 18 days after N.D.V. inoculation.

Infectivity Tests: Quantitative infectivity tests were carried out with the fluids of all the rabbit and chicken tissue cultures 72 hours after N.D.V. inoculation. In the rabbit and chicken tissue cultures which showed hemagglutination titers of 32 to 64 the LD_{50} titers ranged from 10^{-5} to 10^{-7} (Figs. III-VII). In the immune chicken liver cultures the hemagglutinating activity appeared only in the undiluted fluids but an LD_{50} titer of 10^{-3} was found (Fig. IIIb). Although the immune rabbit tissue cultures did not show hemagglutinating activity, LD_{50} titers of 10^{-1} and 10^{-2} appeared within 72 hours after N.D.V. inoculation (Figs. V-VII).

Infectivity tests on the fluids of the normal rabbit and chicken tissue cultures were positive up to 18 to 24 days after N.D.V. inoculation. The immune chicken lung and spleen cultures also showed N.D.V. infectivity during this period but the immune chicken liver and all the tissue cultures of the immune rabbit failed to show this activity after the first fluid change, i.e., four days after N.D.V. inoculation.*

C. Changes in the Tissue Cultures Which Were Infected with N.D.V.

The presence of phenol red in the nutrient medium made it possible to check the pH of the fluids visually. The hydrogen ion concentration of the fluids bathing the tissue cultures did not change after N.D.V. inoculation except in the spleen tissue cultures. The N.D.V.-infected spleen

*Fluid samples from all the tissue cultures were removed each day for four days after N.D.V. inoculation and every other day for the next 14 days for quantitative infectivity tests. These samples were stored at -20° C but before the experiment was completed the freezing unit went out of commission and the infectious activity of the fluids was decreased so that quantitative results could not be obtained. It was necessary, therefore, to repeat the experiment and run only the quantitative infectivity tests which were necessary to confirm the results of the hemagglutination tests. Thus quantitative infectivity tests were carried out on the fluids removed from the embryonating chicken cultures at intervals of 2, 24, 48, 72 and 96 hours after N.D.V. inoculation and on all the other tissue cultures 72 hours after N.D.V. inoculation. The undiluted fluids removed from these cultures 72 hours after each nutrient change were tested for infectious activity but quantitative tests were not carried out.

cultures showed acid production even after two fluid changes but they were less acid than the non-infected spleen controls.

The embryonating chicken tissue cultures showed a decided cytological destruction after N.D.V. inoculation (Plates I-II). The only cytological change in the adult rabbit and chicken tissue cultures occurred in the liver cultures of the normal chicken which were embedded in chicken plasma and this did not take place until seven or eight days after the N.D.V. inoculation. There was a gradual degenerative change in these liver cells in which there was a breaking up of the cytoplasm into large granules and a fragmenting of the cell wall. The entire cytoplasm lost its staining properties but the nucleus remained unaltered. The liver tissues of the normal chicken which were embedded in rabbit plasma and the liver tissues of the immune chicken as well as those of the normal and immune rabbit failed to show any degeneration after N.D.V. inoculation (Plate IV).

None of the control tissue cultures or the tissue cultures inoculated with heat-inactivated N.D.V. showed any degenerative change.

In the embryonating chicken cultures which were embedded in chicken plasma the new cells surrounding the tissue transplant showed definite cytological changes within 15 hours after N.D.V. inoculation. There was an increase in the vacuolization of the cytoplasm of the fibroblasts and

a tendency toward the formation of giant cells which contained three to five nuclei (Pl. I, Figs. 2-3). Sometimes it was difficult to determine whether these multinucleated cells were giant cells or an agglomeration of fibroblasts which had lost their individual cell boundaries and become fused. It was necessary, therefore, to examine these particular cells microscopically every few hours. When several tissue culture fields were marked and examined in this manner it could be seen that the multiple nuclei appeared within a single cell. By 48 hours a pronounced degeneration occurred in the fibroblasts surrounding the tissue culture explants (Pl. II, Figs. 5-6). The cytoplasm became more vacuolated and filled with fatty globules, the cell membrane lost its continuity and most of the fibroblasts almost lost their staining properties. In some areas only shrunken pyknotic nuclei were left. Within four or five days degeneration of all the fibroblasts surrounding the tissue explant occurred leaving only a fringe-like halo of faintly stained ghost cells. A few scattered round cells remained.

The infected embryonating tissue cultures which were embedded in rabbit plasma showed the same type of degeneration but this process took place at a slower rate, lagging 24 to 48 hours behind the degenerative changes shown by the tissues embedded in chicken plasma (Pl. II, Figs. 4, 6). The embryonating tissues which were embedded

in rabbit plasma seemed to be protected in some manner by the rabbit plasma. Only about 50 percent of the fibroblasts surrounding the tissue explant showed degeneration, and many of the remaining cells looked normal even 14 days after N.D.V. inoculation. There was a higher proportion of round cells produced by the embryonating tissues embedded in rabbit plasma and these cells showed no cytological change after exposure to the virus (Pl. II, Fig. 6). Multinucleated giant cells appeared also in these tissues (Pl. II, Fig. 4). There were never more than two nuclei found in any of the fibroblasts arising from the embryonating chicken cultures which did not receive the N.D.V. inoculum, or in the tissues which received the heat-inactivated N.D.V. suspension.

EXPLANATION OF FIGURES III - VII

H.A = hemagglutination titers

↓ = nutrient fluid change

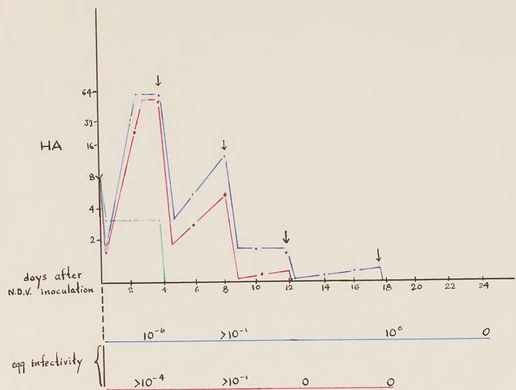
_._ = period of hemagglutination titration

——— (black line) = hemagglutination or infectivity titers of active N.D.V. in normal chicken or rabbit tissue cultures

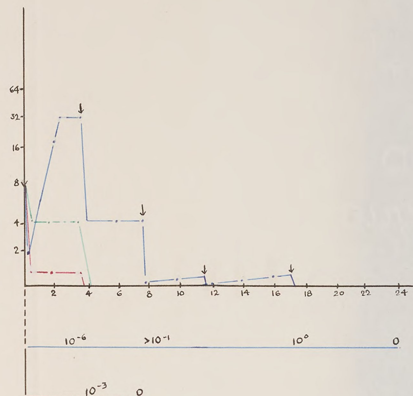
——— (red line) = hemagglutination or infectivity titers of active N.D.V. in immune chicken or rabbit tissue cultures

——— (green line) = hemagglutination titers of heat-inactivated N.D.V. in immune chicken or rabbit tissue cultures

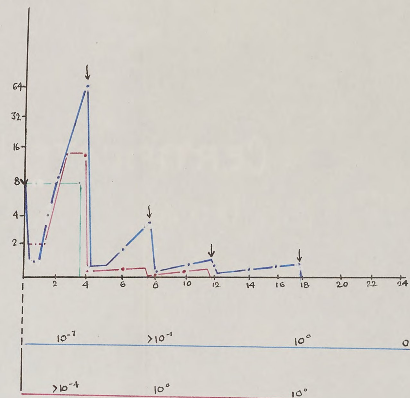
Note: H.A. of heat-inactivated N.D.V. in normal chicken and normal rabbit tissues were omitted in the Figures because the titer remained constant at 8 and decreased to zero after the first nutrient fluid change



IIIa NORMAL AND IMMUNE CHICKEN LUNG CULTURES



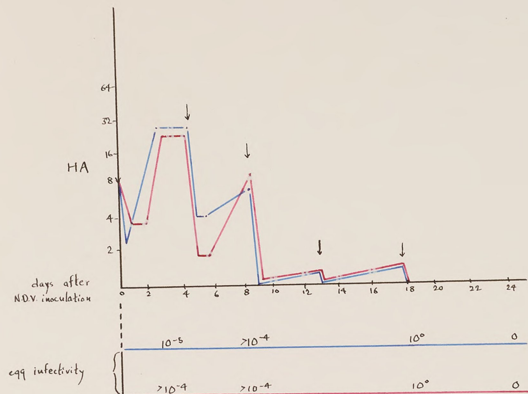
IIIb NORMAL AND IMMUNE CHICKEN LIVER CULTURES



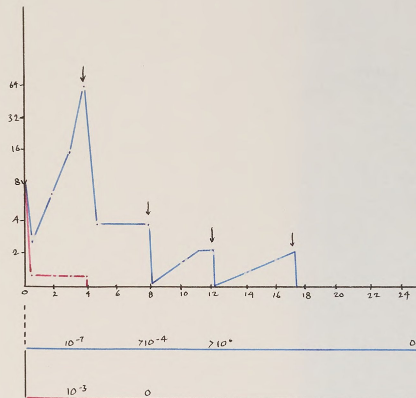
IIIc NORMAL AND IMMUNE CHICKEN SPLEEN CULTURES

HEMAGGLUTINATION AND INFECTIVITY TITERS OF ACTIVE AND INACTIVE N. D. V. IN NORMAL AND IMMUNE CHICKEN TISSUES EMBEDDED IN CHICKEN PLASMA

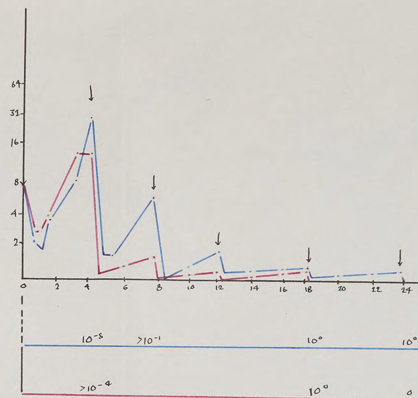
FIG. III



IV a NORMAL AND IMMUNE CHICKEN LUNG CULTURES



IV b NORMAL AND IMMUNE CHICKEN LIVER CULTURES

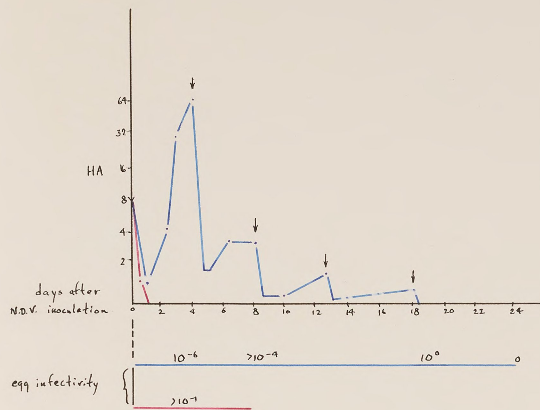


IV c NORMAL AND IMMUNE CHICKEN SPLEEN CULTURES

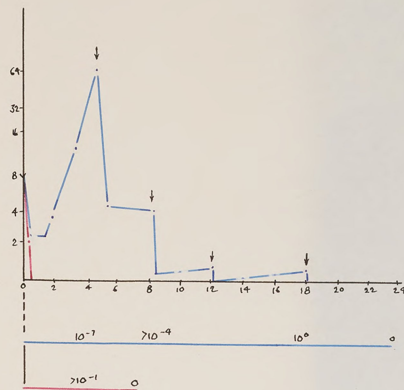
HEMAGGLUTINATION AND INFECTIVITY TITERS OF ACTIVE AND INACTIVE N. D. V. IN NORMAL AND IMMUNE CHICKEN TISSUES

EMBEDDED IN RABBIT PLASMA

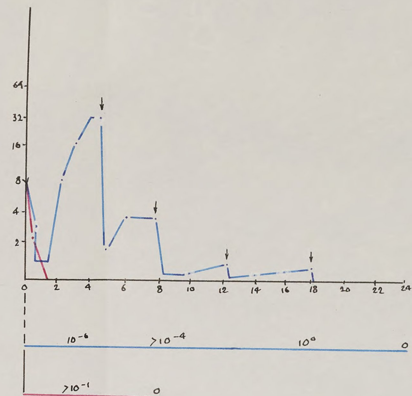
FIG. IV



Ⅴa NORMAL AND IMMUNE RABBIT LUNG CULTURES



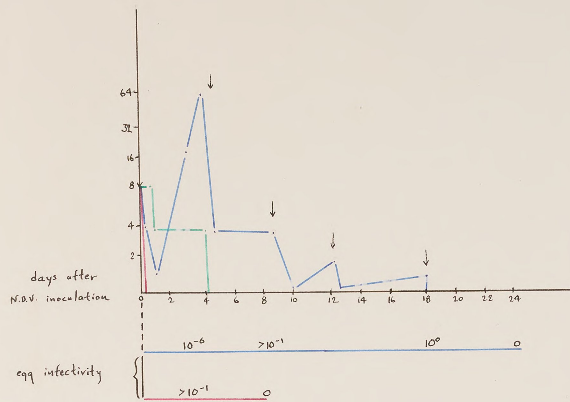
Ⅴb NORMAL AND IMMUNE RABBIT LIVER CULTURES



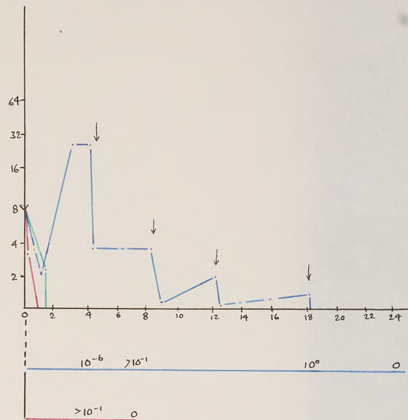
Ⅴc NORMAL AND IMMUNE RABBIT SPLEEN CULTURES

HEMAGGLUTINATION AND. INFECTIVITY TITERS OF ACTIVE AND INACTIVE N. D. V. IN NORMAL AND IMMUNE RABBIT TISSUES EMBEDDED IN RABBIT PLASMA

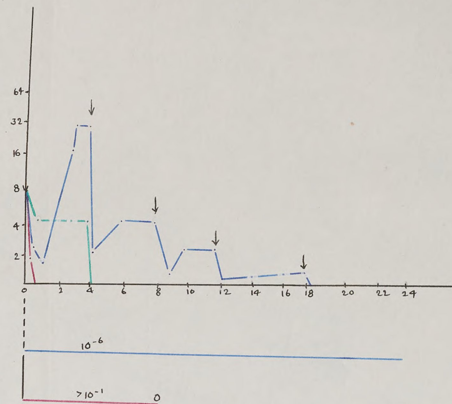
FIG. Ⅴ



VIa NORMAL AND IMMUNE RABBIT LUNG CULTURES



VIb NORMAL AND IMMUNE RABBIT LIVER CULTURES

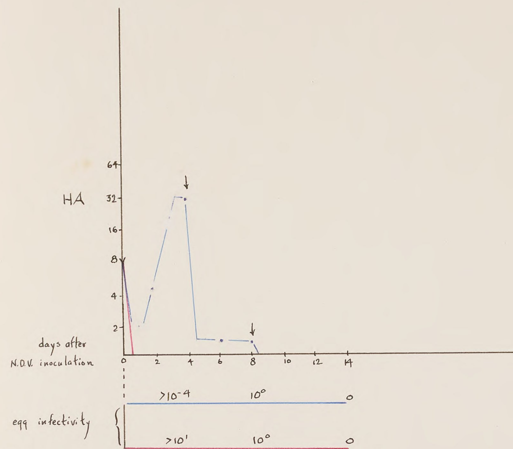


VIc NORMAL AND IMMUNE RABBIT SPLEEN CULTURES

HEMAGGLUTINATION AND INFECTIVITY TITERS OF ACTIVE AND INACTIVE N. D. V. IN NORMAL AND IMMUNE RABBIT TISSUES EMBEDDED IN CHICKEN PLASMA

FIG. VI

FIG. VII



HEMAGGLUTINATION AND INFECTIVITY TITERS IN NORMAL AND IMMUNE RABBIT LYMPH NODE CULTURES EMBEDDED IN RABBIT PLASMA

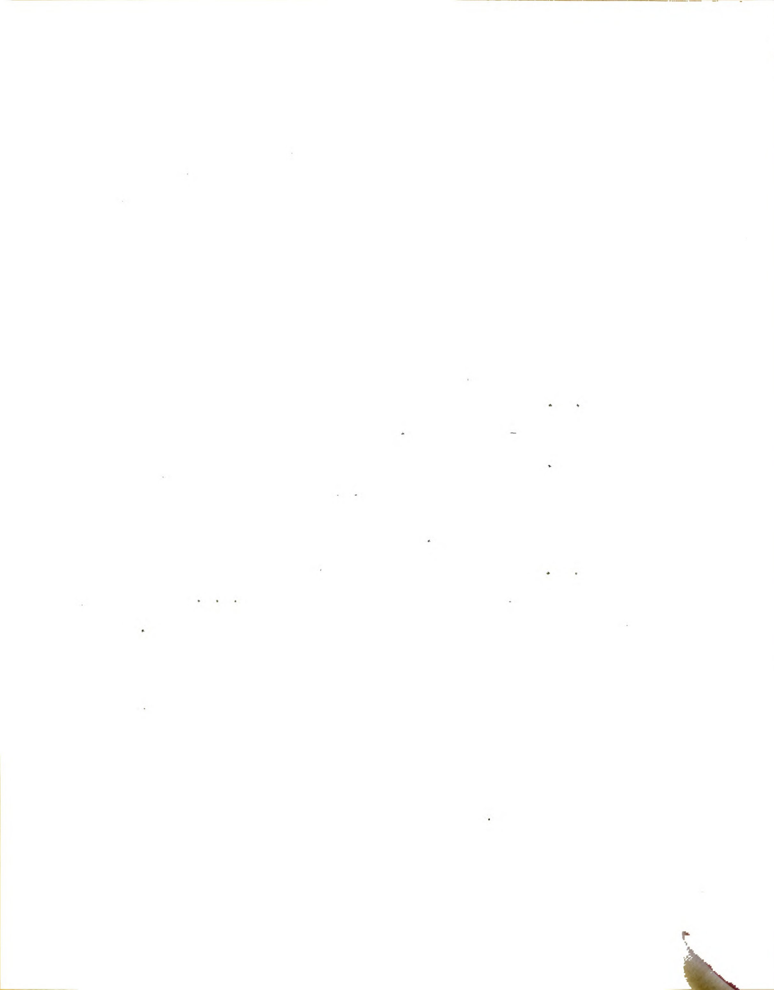


PLATE I

Results of Experiment with Embryonating Chicken Tissue Cultures

Fig. 1. Normal embryonating chicken fibroblasts embedded in chicken plasma matrix, 6-day culture.

Fig. 2. Embryonating chicken fibroblasts embedded in chicken plasma matrix, 5-day culture, 15 hours after N.D.V. inoculation, showing many normal fibroblasts, a few multinucleated fibroblasts and one multinucleated giant cell.

Fig. 3. Embryonating chicken fibroblasts and round cells embedded in chicken plasma matrix, 5-day culture, 24 hours after N.D.V. inoculation, showing an increase in vacuolization in cytoplasm of fibroblasts.

Magnification: 200x

PLATE I

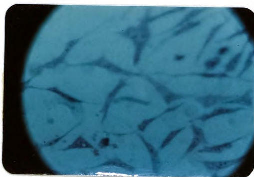


Fig. 1

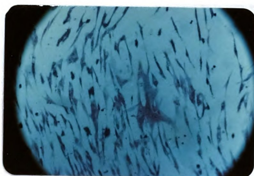


Fig. 2

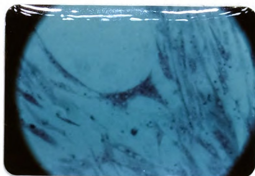


Fig. 3

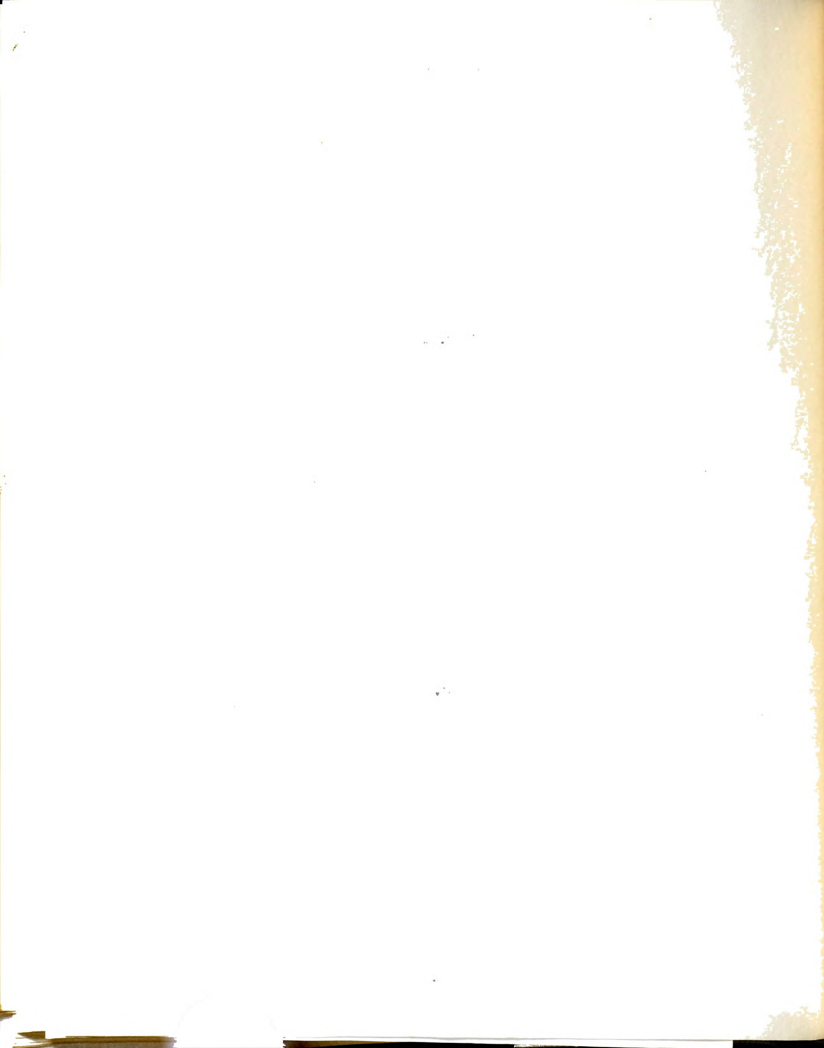


PLATE II

Results of Experiment with Embryonating Tissue Cultures

Fig. 4. Embryonating chicken fibroblasts and round cells, embedded in rabbit plasma, 5-day culture, 24 hours after N.D.V. inoculation, showing a large multinucleated giant cell.

Fig. 5. Embryonating chicken tissue culture, embedded in chicken plasma, 6-day culture, 48 hours after N.D.V. inoculation, showing complete degeneration of fibroblasts and abnormal round cells.

Fig. 6. Embryonating chicken tissue culture, embedded in rabbit plasma, 8-day culture, 96 hours after N.D.V. inoculation, degeneration of fibroblasts not complete, many normal round cells.

Magnification: 200x

PLATE II

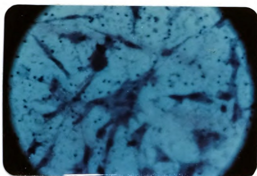


Fig. 4

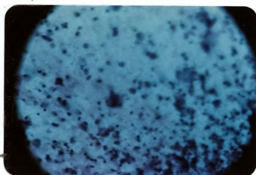


Fig. 5

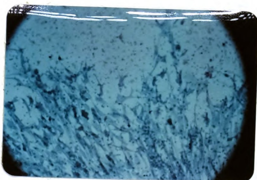


Fig. 6

PLATE III

Cultures of Adult Chicken and Rabbit Tissue

Fig. 7. Normal adult rabbit lung, 8-day culture, normal alveolar cells.

Fig. 8. Normal adult chicken lung, 8-day culture, 96 hours after N.D.V. inoculation showing normal-appearing alveolar cells.

Fig. 9. Normal adult rabbit spleen, 8-day culture, 96 hours after N.D.V. inoculation showing normal-appearing spleen cells.

Magnification: 200x

PLATE III

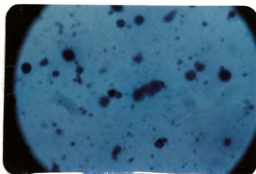


Fig. 7

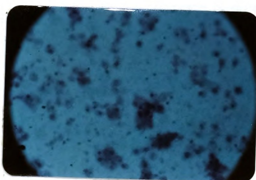


Fig. 8

Fig. 9

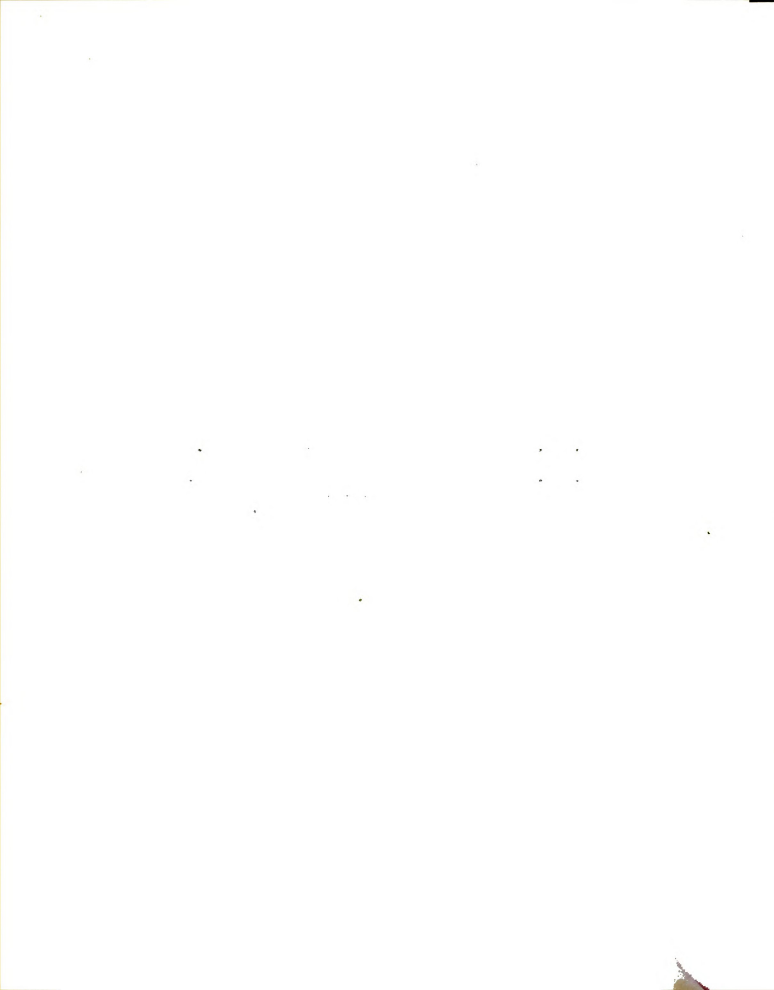


PLATE IV

Fig. 10. Normal rabbit liver, 8-day culture.

Fig. 11. Immune chicken liver, 8-day culture, 96 hours after N.D.V. inoculation showing normal-appearing liver cells.

Magnification: 200x

PLATE IV

Fig. 10

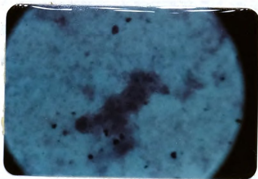


Fig. 11

DISCUSSION

The data from this study were compiled from hemagglutination tests and microscopic studies from three experiments including cultures of embryonating chicken tissue, normal chicken and rabbit tissue and immune chicken tissues and from two experiments with cultures of immune rabbit tissue. In the first experiment quantitative infectivity tests and hemagglutination tests were carried out with the fluids removed from the embryonating chicken cultures at intervals of 2, 6, 12, 24, 36, 48, 72 and 96 hours after N.D.V. inoculation in order to prepare the N.D.V. growth curves (Fig. II). At the same time it was possible to determine the period in which the infectious activity and hemagglutinating activity appeared in the tissue culture fluids. In this experiment fluids from the cultures of tissues from the normal chicken and rabbit and the immune chicken were removed 72 hours after N.D.V. inoculation to determine their LD_{50} titers also, and to compare these titers with the hemagglutination titers. Quantitative infectivity tests were also carried out with the fluids removed 12 hours after N.D.V. inoculation from the tissues of the immune rabbit, which were embedded in chicken plasma.

From the data obtained from the hemagglutination tests and infectivity tests it was found that the growth curves

of N.D.V. in the embryonating chicken tissues cultures showed a cycle similar to that of influenza virus (Enders, 1950). This cycle is composed of (a) a lag phase which occurred within two to 24 hours after virus inoculation when the virus was adsorbed by the tissue culture cells, (b) a phase of rapid virus multiplication in which peak infectivity titers were reached by 48 hours and peak hemagglutination titers within 72 hours, (c) a narrow plateau lasting for 24 hours, and (d) a sudden decrease in virus production. These growth curves of the G.B. strain of N.D.V. were similar to those determined for the Roakin strain of N.D.V. in chorio-allantoic tissue cultures by Scott (1953). The peak infectivity and hemagglutination titers for the G.B. strain of N.D.V. in the embryonating chicken tissue cultures were slightly lower. However, lower titers were expected, for it has been shown that the chorio-allantoic membrane yields a higher N.D.V. content than embryo tissue when the embryonating chicken egg is inoculated by the intravenous route (Hanson _et al_., 1952). The appearance of hemagglutinating activity 24 hours later than infectious activity was also found by Gordon (1952) when the embryonating chicken egg was inoculated with N.D.V. The rate of multiplication of the virus was slower and the hemagglutination and infectivity titers were lower in the tissue cultures.

The fact that there was little difference in the peak

hemagglutination titers and infectivity titers found in the fluids of tissue cultures embedded in rabbit and chicken plasma indicates that these body fluids are not associated with the resistance or susceptibility of the animal as far as multiplication of N.D.V. is concerned. The rabbit plasma, however, showed protection of the N.D.V.-infected liver tissues of the normal chicken and prevented extensive degeneration of the embryonating chicken fibroblasts, although it did not impair their ability to support virus growth. Once the fibroblasts were destroyed in the embryonating chicken cultures, which were embedded in chicken plasma, there was a sharp decrease in virus multiplication and the virus activity found in these tissue culture fluids eight days after N.D.V. inoculation could have arisen only from a few viable cells in the explant or the remaining round cells. Since there were a considerable number of fibroblasts and round cells found in the embryonating chicken tissues embedded in rabbit plasma 12 days after N.D.V. inoculation, the rabbit plasma apparently spared these cells so that the N.D.V. growth curve showed a slower decrease in virus multiplication as well as an extension of the period of virus multiplication. If degenerative changes did not occur in the tissue cultures of the normal chicken and rabbit their capacity to support growth of the virus was not exhausted until 12 to 18 days after N.D.V. inoculation.

The inhibition of growth of N.D.V. in the liver tissue

cultures of the immune chicken and all the tissues cultured from the immune rabbit was due evidently to a property acquired by the tissues themselves during the period of immunization because the fluids extracted from these tissues did not show inhibition of the virus at the time they were removed from the animal. The fact that the spleen tissue cultures of the immune chicken showed only slight inhibition of virus multiplication may be explained on the basis that the multiplication of the spleen cells continued to increase at a much greater rate than that of the liver cells after N.D.V. inoculation and there might have been a higher percentage of new spleen cells which had not acquired the N.D.V. inhibiting property. On the other hand, it could have been due to the fact that the spleen of the immune chicken does not play as important a role in the process of immunization as does the liver.

Since all the tissues cultured from the immune rabbit failed to support growth of N.D.V. to the same extent as the tissues cultured from the normal chicken, and rabbit as well as the lung and spleen tissues of the immune chicken tissue the resistance of the rabbit to N.D.V. disease may be due to the fact that the rabbit has many more tissues which are capable of acquiring a N.D.V.-inhibiting property when the animal is exposed to the virus although the rabbit may not be able to produce an antibody titer any higher than that of the chicken. There is also the additional

possibility that when the rabbit is first exposed to N.D.V. it may possess a plasma activity capable of protecting the tissues so that degeneration of the cells may be prevented until there are sufficient specific antibodies produced against the virus to stop the process. In this case multiplication of the virus in the rabbit tissues occurs without production of disease.

Although the growth curve of N.D.V. in tissue cultures was similar to that of influenza virus it is of interest to note that influenza produced degeneration of the cells of the embryonating chicken cultures (Enders, 1950). Mumps virus did not produce cellular changes (Enders, 1952) but this was not expected because this virus does not produce the same degree of damage in the embryonating chicken egg as do influenza virus and N.D.V. Newcastle disease virus, instead, seems to resemble poliomyelitis virus in its ability to destroy tissue culture fibroblasts. The production of multinucleated giant cells in embryonating chicken cultures inoculated with N.D.V. may be of diagnostic significance for these cells appear within 12 to 24 hours after exposure to the virus. The possibility that the tissue culture technique could provide a simple and rapid means for identifying N.D.V. warrants further investigation. The occurrence of multinucleated giant cells has often been reported in tissue cultures of malignant tumors but never in tissue cultures infected with a virus. Burnet (1942)

described the appearance of "large, vacuolated cells, agglutinated in masses in which it is impossible to pick out the outline of individual cells" in tracheal smears of the N.D.V.-infected embryonating chicken egg. It is possible that what would appear as an aggregation of superimposed cells in a smear preparation could have been one single multinucleated giant cell. Otherwise the production of multinucleated cells has not been reported in connection with N.D.V. When microscopic examination can be made on a single layer of cells which can be obtained in tissue culture preparations any changes in the isolated cells can be followed more easily.

SUMMARY

In these experiments a comparative study has been made on the effect of N.D.V. on susceptible and resistant tissues cultured from chickens and rabbits.

Embryonating chicken, adult chicken lung, liver and spleen and adult rabbit lung, liver, spleen and lymph node tissues were cultured. Both normal and immune adult chickens and rabbits were used. Hemagglutination and infectivity tests were carried out to determine the growth curves of N.D.V. in embryonating chicken tissues.

The growth cycle was composed of (a) a lag phase of 24 hours, (b) a phase of rapid virus multiplication in which peak infectivity titers were reached in 36 hours and peak hemagglutination titers in 36 to 48 hours, (c) a plateau lasting 48 to 60 hours, (d) a sudden decrease in the rate of virus production 96 hours after N.D.V. inoculation.

Growth curves prepared from hemagglutination titrations on the tissues cultured from normal adult chickens and rabbits were similar to those of the embryonating chicken tissue but the peak hemagglutination titers occurred 36 to 96 hours after N.D.V. inoculation. This was also found in the immune chicken lung and spleen cultures but not in the liver cultures. In the immune chicken liver tissues and in all the tissues of the immune rabbit there was a steep

decrease in infectivity titers from an LD_{50} of 10^{-7} on the day of inoculation to 10^{-1} or 10^{-2} 48 hours later. Hem-agglutination activity was recovered only in the undiluted fluids from the immune chicken liver cultures.

Cytological degeneration was seen in the embryonating chicken cultures 15 to 48 hours after N.D.V. inoculation. The fibroblasts showed an increase in vacuolization of the cytoplasm and a tendency in the formation of giant cells which contained three to five nuclei. The only degenerative changes in the adult tissue cultures occurred in normal adult chicken liver and this did not appear until seven or eight days after N.D.V. inoculation. There was a gradual degeneration of the liver cells in which the cyto-plasm lost its staining properties and broke up into large granules and the cell wall became fragmented. The nucleus, however, was unaltered. Neither the uninoculated cultures nor the cultures inoculated with heat-inactivated N.D.V. showed any degenerative change.

BIBLIOGRAPHY

Bankowski, R. A. and Boynton, W. H. 1948. A preliminary report on the propagation of avian pneumoencephalitis virus (N.D.V.) in vitro. *Vet. Med.* 43(7):305-306.

Bang, F. B. 1946. Filamentous forms of Newcastle disease virus. *Proc. Soc. Exp. Biol. Med.* 63:5-7.

Beaudette, F. R. 1943. A review of the literature on Newcastle disease. *Proc. 47th Ann. Meet. U.S. Livestock San. Assn.*, 122-177.

Bland, J. D. S. and Canti, R. G. 1935. The growth and development of psittacosis virus in tissue cultures. *J. Path. Bact.* 40:231-241.

Brandly, C. A., Moses, H. E., Jungherr, E. L. and Jones, E. E. 1946a. Epizootiology of Newcastle disease of poultry. *Amer. J. Vet.* Res. 7:243-249.

Brandly, C. A., Moses, H. E., Jungherr, E. L. and Jones, E. E. 1946b. Isolation and identification of Newcastle disease virus. *Amer. J. Vet. Res.* 7:284-306.

Brandly, C. A., Moses, H. E., Jungherr, E. L. and Jones, E. E. 1946c. Immunization of chickens against Newcastle disease. *Amer. J. Vet. Res.* 7:307-332.

Brandly, C. D., Moses, H. E., Jungherr, E. L. and Jones, E. E. 1946d. Transmission of antiviral activity via the egg and the role of congenital passive immunity to Newcastle disease in chickens. *Amer. J. Vet. Res.* 7:333-342.

Brandly, C. A., Hanson, R. P., Lewis, S. H. and Winslow, N. S. 1947. Variables and correlations in laboratory procedures for Newcastle disease diagnosis. *Cornell Vet.* 37:324-336.

Burnet, F. M. and Ferry, J. D. 1934. The differentiation of the viruses of fowl plague and Newcastle disease: Experiments using the technique of chorio-allantoic membrane inoculation of the developing egg. *Brit. J. Exp. Path.* 15:56-64.

Burnet, F. M. 1942. The affinity of Newcastle disease virus to the influenza virus group. Aus. J. Exp. Biol. Med. Sci. 20:81-88.

Burnet, F. M., Beveridge, W. I. B., McEwin, J. and Boake, W. C. 1945. Studies on the Hirst hemagglutination reaction with influenza and Newcastle disease viruses. Aus. J. Exp. Biol. Med. Sci. 23:177-192.

Burnet, F. M. and Anderson, S. G. 1946. Modification of human red cells by virus action. II. Agglutination of modified human red cells by sera from cases of infectious mononucleosis. Brit. J. Exp. Path. 27:236-241.

Bureau of Animal Industry. 1946. The hemagglutination and hemagglutination-inhibition tests for the diagnosis of Newcastle disease. Mimeo. Circular, Oct. 21.

Boney, W. A. 1951. Isolation of a neurotropic strain (GB) of Newcastle disease virus. Southwestern Veterinarian V(1):19-21.

Cunha, R., Weil, M. L., Beard, O., Taylor, A. R., Sharp, D. G. and Beard, J. W. 1947. Purification and characters of the Newcastle disease virus (California strain). J. Immun. 55:62-89.

Durusan, R. 1949. Thermal inactivation of Newcastle disease virus. M.S. Thesis, Michigan State College.

Enders, J. F. 1950. Tissue culture techniques in the study of animal viruses. Amer. J. Med. Sci. 220:316-338.

Enders, J. F., Weller, T. H. and Robbins, G. C. 1949. Cultivation of the Lansing strain of poliomyelitis virus in cultures of various human embryonic tissues. Science 109:85-87.

Fukushima, S., Shimomaru, K. and Oyama, S. 1932. Pathologische Anatomie der durch ein filtierbares Virus versuchtachten Huhnersenche in Korea. J. Jap. Soc. Vet. Sci. 9(4):302-316. Cited by Beaudette (1943).

Gordon, L. E., Jorgen, M. B. and Dodd, M. C. 1952. Growth of Newcastle disease virus in the embryonating egg. Proc. Soc. Exp. Biol. Med. 80:205-210.

Gottlieb, O., Bhattacharyya, P. K., Carter, H. E. and Anderson, H. W. 1951. Endomycin, a new antibiotic. Phytopathology XLI(5):393-400.

Hanson, R. P., Winslow, N. S. and Brandly, C. A. 1947. Influence of the route of inoculation of Newcastle disease virus on selective infection of the embryonating egg. Amer. J. Vet. Res. 8:416-420.

Hirst, G. K. 1941. The agglutination of red cells by allantoic fluids of chicken embryos infected with influenza. Science 94:22-23.

Hirst, G. K. 1942a. The quantitative determination of influenza virus and antibodies by means of red cell agglutination. J. Exp. Med. 75:49-64.

Hirst, G. K. 1942b. Adsorption of influenza virus hemagglutinins and virus by red blood cells. J. Exp. Med. 76:195-209.

Hofstad, M. S. 1951. A quantitative study of N.D.V. in tissues of infected chickens. Amer. J. Vet. Res. XII(45): 334-339.

Huang, C. H. 1943a. Further studies on the titration and neutralization of the Western strain of equine encephalomyelitis virus in tissue culture. J. Exp. Med. 78:111-126.

Huang, C. H. 1943b. Titration of St. Louis encephalitis virus and the Jungeblut-Sanders mouse virus in tissue culture. Proc. Soc. Exp. Biol. Med. 54:158-160.

Jungherr, E. L., Tyzzer, E. E., Brandly, C. A. and Moses, H. E. 1946. The comparative pathology of fowl plague and Newcastle disease. Amer. J. Vet. Res. 7:250-288.

Jungherr, E. and Terrell, N. 1946. Observations on the spread of Newcastle disease. Proc. 50th Ann. Meet. U.S. Livestock San. Assn., 158-171.

Jungherr, E., Lungibuhl, R. G. and Kilham, L. 1949. Serological relationships of mumps and Newcastle disease virus. Science 110:333-334.

Karzon, D. T. and Bang, F. B. 1951. The pathogenesis of infection with a virulent (CG 179) and an avirulent (B) strain of Newcastle disease virus in the chicken. I. Comparative rate of viral multiplication. J. Exp. Med. 93(3):267-284.

Levens, J. H. and Enders, J. F. 1945. The hemagglutinative properties of amniotic fluid from embryonated eggs infected with mumps virus. Science 102:117-120.

McClelland, L. and Hare, R. 1941. The adsorption of influenza virus by red cells and a new in vitro method of measuring antibodies for influenza virus. Can. Public Health J. 32:530-538.

Miller, G. L. and Stanley, W. M. 1944. Quantitative aspects of the red blood cell agglutination test for influenza virus. J. Exp. Med. 79:185-195.

Parker, F., Jr. and Nye, R. N. 1925. Studies on filterable viruses. I. Cultivation of vaccine virus. Amer. J. Path. 1:325-335.

Reagan, R. L., Little, M. G., Poelna, L. J. and Brueckner, A. L. 1947a. Transmission of the virus of Newcastle disease virus to the Syrian hamster. Amer. J. Vet. Res. 8:126-138.

Reagan, R. L., Little, M. G., Poelna, L. J. and Brueckner, A. L. 1947b. The response of some mammals to Newcastle disease. Amer. J. Vet. Res. 8:427-430.

Reagan, R. L., Little, M. G., Hanser, J. E., Poelna, L. J. and Brueckner, A. L. 1947c. Response of monkeys to poliomyelitis after infection with Newcastle disease virus. Proc. 51st Ann. Meet. Livestock San. Assn., 55-62.

Reagan, R. L. and Brueckner, A. L. 1952. Response of Syrian hamster to rectal instillations of Newcastle disease virus. Amer. J. Vet. Res. 13:

Reed, L. J. and Muench, H. 1938. A simple method of fifty percent endpoints. Amer. J. Hyg. 27:493-497.

Robbins, F. C., Enders, J. F. and Weller, T. H. 1950. Cytopathogenic effects of poliomyelitis viruses in vitro on human embryonic tissues. Soc. Exp. Biol. Med. 75: 370-374.

Salk, J. E. 1944. A simplified procedure for titrating hemagglutinating capacity of influenza virus and the corresponding antibody. J. Immun. 49:87-98.

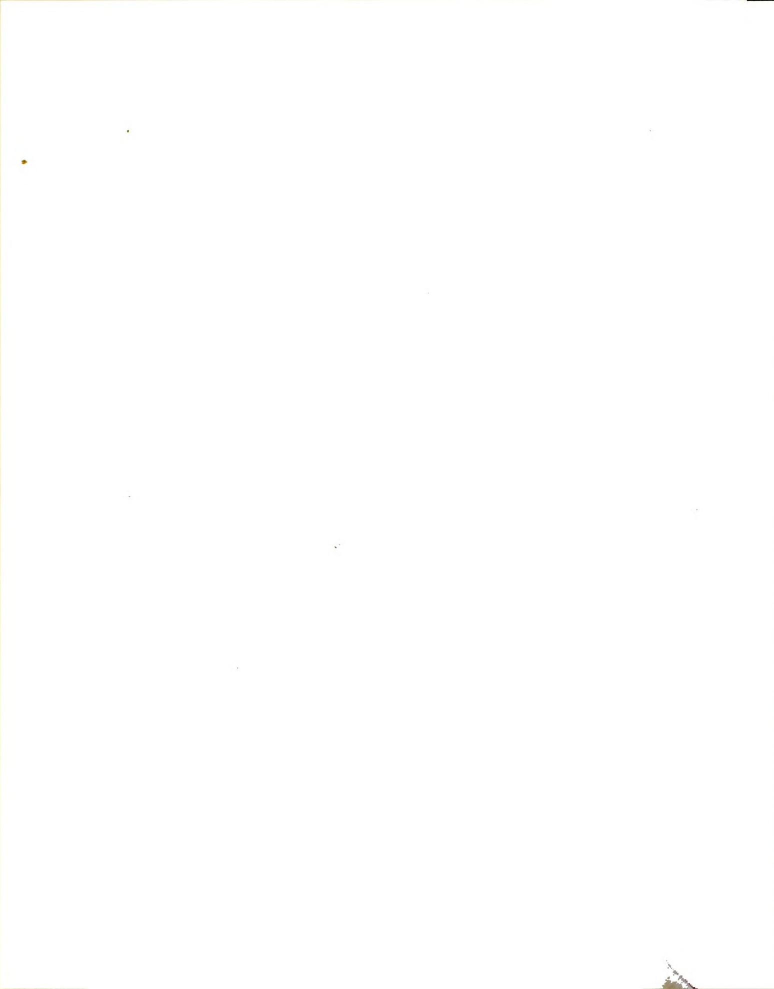
Salk, J. E. 1951. Isolation and serologic identification of poliomyelitis virus strains in tissue cultures from patients with non-paralytic and paralytic poliomyelitis. Amer. J. Hyg. 54:286-293.

Syverton, J. T. and Scherer, W. F. 1952. Studies on the propagation in vitro of poliomyelitis virus. J. Exp. Med. 96:355-389.

Scherer, W. F., Syverton, J. T. and Gey, G. O. 1953. Studies on the propagation in vitro of poliomyelitis viruses. IV. Viral multiplication in a stable strain of human malignant epithelial cells (strain Hela) derived from an epidermoid carcinoma of the cervix. J. Exp. Med. 97:697-710.

Scott, G. R., Hanson, R. P. and Brandly, C. A. 1954. A simplified procedure for the in vitro propagation of Newcastle disease virus. Proc. Amer. Vet. Med. Assn., 19th Ann. Meet. 312-315.

Topacio, T. and Coronel, A. C. 1939. Studies on avian pest. Phillipine J. Animal Ind. 6:467-476.



ROOM USE ONLY

Mar 31 '55
Apr 20 '55
May 5 '55

ROOM USE ONLY

MICHIGAN STATE UNIVERSITY LIBRARIES
3 1293 03062 3171