A STUDY OF THE SERUM NEUTRALIZATION TEST FOR INFECTIOUS BRONCHITIS OF CHICKENS

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This is to certify that the

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A Study of the Serum Neutralization Test

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Calvin Ames Page

has been accepted towards fulfillment
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H. J. Stafseth
Major professor

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A STUDY OF THE SERUM NEUTRALIZATION TEST FOR INFECTIOUS BRONCHITIS OF CHICKENS

By

CALVIN AMES PAGE

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

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THESIS

1-6-56

TO MY WIFE,

LAURIE S., THIS MANUSCRIPT

IS MOST AFFECTIONATELY DEDICATED

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INTRODUCTION

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

The object of the present study is to obtain fundamental knowledge of antibody-antigen reactions with respect to their practical applications to the serum neutralization test for infectious bronchitis.

HISTORICAL REVIEW

Infectious Bronchitis of Chickens

In 1931, Schalk and Hawn[76] described a new respiratory disease occurring in baby chicks and suggested the name "infectious bronchitis". Since that time the disease has been reported throughout the United States[5,6,19,43], Canada[7], England[3,8], and the Netherlands[79].

Etiological Agent

Infectious bronchitis (IB) is caused by a distinct filterable virus capable of passing through all grades of Berkefeld and Seitz filters[9,12,34,35,45,76] and the Mandler preliminary filter[34]. The disease is apparently limited to chickens[83], and the virus is found primarily in the tissues and exudates of the respiratory system[9]. Komarov and Beaudette[58] were unable to find the virus in the liver, spleen, kidney or blood of infected chickens. However, Bushnell and Brandly[19] reported successful transmission of the disease using those tissues. Electron micrographs of infectious bronchitis virus (IBV) indicate that the virus is round with filamentous projections, having a mean diameter of 70 millimicrons[73,74]. The virus will remain viable for 180 days if lyophilized and stored at 4°C, and for 80 days if glycerolized and refrigerated at 4°C[9].

Transmission

IBV is highly infectious for chickens and can produce the disease in all ages within 24 to 48 hours[6,31,34,83]. Aerosol transmission cannot be controlled by ultraviolet irradiation of the air[61]. The virus can be readily transmitted by intratracheal and intranasal inoculation[12,34], but subcutaneous and intramuscular inoculations fail to produce the disease[12]. The virus can be isolated from the yolks of eggs laid between the second and thirty-sixth post-inoculation day, and it can be recovered from tracheal swabs as late as four weeks after inoculation[40]. Komarov and Beaudette[58] found carriers of the virus 43 days after an outbreak. Delaplane and Stuart[34] reported that recovered chickens can be carriers for at least two months. Hofstad[46,48] demonstrated that chickens could transmit the disease for 35 days after recovery.

Symptoms

Characteristic symptoms of IB include gasping, sneezing, coughing and tracheal rales[9,12,31,34,76]. Nasal discharges are noted in 30 to 50 per cent of the cases[31]. The outstanding lesions are mucous, catarrhal and purulent accumulations in the trachea and bronchi accompanied by congestion and edema of the lungs[31,34,45,83,84]. Edema of the facial sinuses may be found in chicks under two to three weeks of age. There is no hemorrhage or significant changes in the liver, spleen and kidney and inclusion bodies are not found[45]. The

severity of the symptoms is dependent upon such predisposing factors as environmental conditions and nutritional deficiencies[83].

The morbidity of IB is high. The highest mortality rate, ranging to as much as 90 to 100 per cent, occurs in young chicks[9,12,31,34,76]. In laying flocks there is a marked decline in egg production that may persist from four to nine weeks[31,34,83]. The first few eggs laid when the flock is returning to production may be misshapen, rough, thin-shelled with watery albumen[83].

Diagnosis

Diagnosis is based on history, clinical symptoms and laboratory tests such as isolation and identification of the virus in embryonating chicken eggs and serum neutralization tests, in conjunction with the characteristic alterations of the chicken embryo[11,12,24,34,35,37,38,63,83]. In addition, IBV does not possess the ability to agglutinate chicken red blood cells as does Newcastle disease virus, and this test may be used for differential diagnosis[38,47].

The serum neutralization test is useful in evaluating flock immunity. Normal chicken serum would not be expected to have more than 36 neutralizing doses[25,39,70,84]. A minimum of one hundred doses is considered as a positive test, and this is usually obtained with serum collected about three weeks following exposure to the virus[39,70].

Cultivation of the Virus in Embryonating Chicken Eggs

It was found by Beaudette and Hudson[12] that the IBV could be propagated in embryonating chicken eggs via the chorioallantoic membrane. After a few passages, death of some of the embryos resulted from virus inoculation. After the fourteenth passage in embryos, the virus was still infectious for chickens. Delaplane and Stuart[34,35] reported similar results and noted that with each succeeding passage the virus became more virulent for the embryo. By the sixty-fifth passage, the virus was completely egg-adapted with respect to embryo mortality and no apparent change was noted with subsequent passage. At the ninetieth passage, the virus had lost its pathogenicity and antigenicity for chickens. According to Beaudette[10], the virus had lost its pathogenicity but not its antigenicity for the natural host after the seventieth passage in eggs. Neutralizing antibodies could be demonstrated fourteen days after inoculation. Adaptation of the virus to embryonating chicken eggs can be accomplished earlier by inoculation via the allantoic cavity rather than the chorioallantoic membrane[27,32].

The outstanding gross alterations of an embryo following inoculation with chicken-propagated virus is curling and dwarfing of the embryo to as much as one-half the normal size. Thinning of the chorioallantoic membrane, thickening of the amnionic membrane, hemorrhage and congestion of the liver, and swelling of the kidney and spleen are observed[11,12,34,35,37].

63,83. In addition, microscopic alterations, as reported by Loomis et al[63], include proliferation of mesodermal and ectodermal cells, edema of the chorioallantoic and amnionic membranes, pneumonia and marked serous exudation, interstitial nephritis and necrosis, splenic congestion and congestion of brain capillaries.

Cunningham and Stuart[30] reported that the completely egg-adapted virus is capable of killing all embryos within a 48-hour post-inoculation period. The highest concentration of the virus, following inoculation via the allantoic cavity, was found in the chorioallantoic membrane, followed in order by the allantoic fluid, amnionic fluid, and the liver according to Cunningham and El Dardiry[26]. The highest titer was obtained at the thirty-sixth hour post-inoculation from living embryos since the virus was thermolabile in eggs incubated at 99°F for eight to twelve hours after the death of the embryo[26].

Groupe[42] demonstrated that infected eggs stored for 24 hours at 36°C after the death of the embryo contained a non-infectious material which adsorbed to and interferred with the infectivity of the virus. According to Groupe and Pugh [43], embryos inoculated with egg-avirulent IBV or influenza A virus would be protected against subsequent inoculation with embryo-lethal IBV.

The egg-adapted strain of IBV has a greater stability in an acid medium than in an alkaline medium during the first

sixty days of storage. After the sixtieth day, the virus is more stable in an alkaline medium[29]. The virus is inactivated by 1 per cent phenol, 1 per cent liquor cresolis saponatus, 1 per cent metaphen, 1:10,000 $KMnO_4$, 1:1,000 $HgCl_2$, 95,70,40 and 25 per cent ethanol, 1:1,000 tincture of Zephiran, 1 per cent Lugol's iodine, 1:20 NaOH, 5 per cent Neoprontosil and 1 per cent formalin in three minutes or less[28]..

Immunity to infectious bronchitis

Chickens recovered from the disease are immune, and neutralizing antibodies can be demonstrated in the blood [6,12,34,46,56]. This immunity persists for at least one year[34], but occasionally flock immunity is inadequate to prevent a natural outbreak[83]. Jungherr and Terrell[56] have reported a naturally acquired passive immunity which may persist in chicks for as long as five weeks after hatching. However, Hofstad and Kenzy[49] reported that four, six, seven and ten-day old chicks hatched from eggs laid by immune hens could be infected by overwhelming challenges with IBV.

Control

A program of active immunization with commercially prepared egg-adapted antigenic virus is used in many regions to control the disease[10,31,33]. Chickens from four weeks to four months of age can be inoculated without untoward results [10,31,33,62,81,82]. However, the best age is from ten to fourteen weeks with exposure being made during June, July

and August when the flock is on range and conditions are most favorable for recovery[82]. The program is generally not started unless 75 per cent of the poultrymen in the community approve the plan. About one per cent of the flock is inoculated and the disease spreads naturally in about three to six days[64].

EXPERIMENTAL PROCEDURES

The study was divided into two parts: (1) thermolability of IBV, and (2) certain factors involved in the serum neutralization test for IB.

In all thermolability and serum neutralization experiments, egg-adapted IBV, strain V114D, was used. This strain of the virus was capable of killing all embryos within 48 hours following inoculation. It was supplied by Dr. Charles H. Cunningham, Department of Bacteriology and Public Health, Michigan State College, East Lansing, Michigan.

IBV, strain VR, in the form of infected allantoic fluid from the first passage of the virus in embryonating chicken eggs was used to inject adult Single Comb White Leghorn cockerels by the intratracheal and intranasal routes for the production of specifically immune serum. This strain was originally isolated by Dr. H. Van Roekel, University of Massachusetts. Serum was collected by cardiac puncture between the sixth and eighth weeks after inoculation.

For studies of time-temperature relations of thermolability of IBV, virus-infected allantoic fluid collected from living embryos at 26 hours post-inoculation was employed. The allantoic fluid was pooled and frozen at -45°C for about one day. The preparation was then thawed at room temperature and centrifuged at 3,5000 r.p.m. at 4°C for 30 minutes to sediment the insoluble precipitate formed by freezing and

thawing. The clear supernatant fluid was removed and distributed into 30 ml. screw cap vials which were then stored at -45°C until ready for use. For uniformity, one vial of fluid was used for each study of thermal inactivation.

At the time of use, the virus was thawed at room temperature and centrifuged. The clear supernatant fluid was distributed into thin-walled, long neck serum ampoules which were sealed by flame. For all studies with the exception of that at 56°C, 2.0cc ampoules containing 1.7cc of virus suspension and 1.0cc ampoules containing 0.7cc of virus suspension were prepared. For the studies at 56°C, only 2.0cc ampoules were prepared.

All ampoules for a particular thermolability test were submerged in a water bath thermostatically controlled at the respective temperatures. At certain time intervals, two ampoules were removed. With the exception of those subjected to 4°C, all ampoules were immersed in an ice bath to stop thermal inactivation of the virus. One ampoule was used for the qualitative infectivity test and the other was stored at -45°C until used for the quantitative infectivity test.

Qualitative infectivity tests were performed at 56, 37, and 22-25°C exposures as screening tests to ascertain the maximum exposure period during which the virus retained some degree of infectivity in order to select suitable

samples for quantitative infectivity tests. The qualitative infectivity tests were made by injecting 0.1cc of the respective virus samples into ten 10-day embryonating chicken eggs. The criterion for inactivation of the virus was failure of the virus to kill the embryo within five days after inoculation.

Quantitative determination of viral infectivity for the thermolability tests was accomplished by preparing serial ten-fold dilutions of the virus-infected allantoic fluid in nutrient broth using separate pipettes per dilution. Five eggs were employed per dilution and each was inoculated with 0.1cc via the allantoic cavity.[22] The eggs were incubated for five days following inoculation. Mortality rates were used in computing the titer which was expressed as the lethal $dose_{50}$ ($l.d._{50}$) according to the method of Reed and Muench[75]. The $l.d._{50}$ was calculated to the centile and rounded-off to the decile. The number of lethal doses of virus was considered to be the antilog of the reciprocal of the $l.d._{50}$.

All incubation of eggs was at 99-99.5°F (86-88°F wet bulb) in an electric, forced-draft incubator. Eggs were candled daily. Embryo mortality during the first 24 hours was considered to be due to nonspecific causes and these eggs were not included in the final results.

Statistical interpretations of the results were made according to the procedures of Baten[4], Croxton[22] and Dixon[36].

The results of the thermolability tests best fit a second-degree parabolic curve of the equation $Y = a + bX + cX^2$ as computed from the following equations:

(1) $Na + b\Sigma X + c\Sigma X^2 = \Sigma Y$

(2) $a\Sigma X + b\Sigma X^2 + c\Sigma X^3 = \Sigma XY$

(3) $a\Sigma X^2 + b\Sigma X^3 + c\Sigma X^4 = \Sigma X^2Y$

The results of the serum neutralization studies best fit a regression line of the equation $Y = a + bX$ as computed from the following equations:

(4) $Na + b\Sigma X = \Sigma Y$

(5) $a\Sigma X + b\Sigma X^2 = \Sigma XY$

Standard deviations were calculated from the following equation:

$$\sigma = \sqrt{\frac{(\Sigma X^2) - \frac{(\Sigma X)^2}{N}}{N - 1}}$$

Variance was calculated from the following equation:

$$v = \frac{(\Sigma X^2) - \frac{(\Sigma X)^2}{N}}{N - 1}$$

The standard error of estimate for Y from the linear regression equation was calculated from the following equation:

$$S_y = \sigma_y \sqrt{1 - r^2}$$

The serum neutralization tests were performed by preparing serial ten-fold dilutions of an allantoic fluid suspension of the virus in nutrient broth. Serum-virus mixtures were prepared in separate tubes by mixing equal parts of the virus dilution and undiluted serum. To compensate for the increased dilution of virus when mixed with serum, each virus dilution was mixed with an equal part of diluent in the same volume as used for the serum-virus mixtures. In all mixtures, 0.5 ml. of each ingredient was used unless otherwise specified. Separate pipettes were used for preparing all dilutions and serum-virus mixtures. The ingredients were incubated at room temperature for about 45 minutes before inoculation unless otherwise specified.

The virus dilutions were inoculated last to take into consideration any possible deleterious effect of prolonged incubation. The inoculum was 0.1 cc. per egg unless otherwise specified. The eggs were reincubated and candled daily for five days. Embryo mortality within the first 24 hours was attributed to nonspecific causes and was not included in the final results.

The fifty per cent end-point formula of Reed and Muench[75], expressed as $l.d._{50}$, was used to evaluate all titrations. The $l.d._{50}$ neutralization index ($l.d._{50}NI$) was the difference between the reciprocals of the virus and the serum titers. The antilog of the $l.d._{50}NI$ represented the number of neutralizing doses (n.d.).

EXPERIMENTAL RESULTS

I. Thermolability of IBV

Schalm and Beach[77] demonstrated that laryngotracheitis virus was completely inactivated at 55.5°C for 15 minutes, 60°C. for three minutes, and 75°C for one-half minute. Shahan[78] found that vesicular stomatitis virus remained infective for five to eight days at 37°C, and for 40 to 52 days at 3°C to 5°C. Amies[1] reported that vaccinia virus remained infectious for as long as 30 days at 37°C and 140 days at 0°C. Beaudette[10] demonstrated that a lyophilized egg-adapted strain of IBV was infective after 2,562 days at 4°C. Cunningham and El Dardiry[26] found that an egg-adapted strain of IBV would retain its initial titer for 30 days when stored at -35°C followed by a ten-fold decrease in titer at 60 days.

Lauffer and Price[59] found that at 69.8°C the thermal inactivation of the tobacco mosaic virus was a first order reaction. Lauffer et al[60] reported that thermal inactivation of influenza A at 45-48°C was likewise a first order reaction.

Nanavutty[67] concluded that resistance of coli-bacteriophage to heat varies with the suspending medium. The coli-bacteriophage was completely inactivated following exposures for three minutes at 65°C when suspended in physiological saline, ten minutes when suspended in a one per cent peptone-water solution, pH 7.8, and thirty minutes when suspended in nutrient broth, pH 7.8.

A. Thermolability at 4°C.

It was not considered necessary to make preliminary qualitative infectivity measurements prior to the quantitative measurements at this temperature as previous experience had shown that the virus would retain its infectivity for about six months.

One week intervals for the first two tests were used and two week intervals thereafter for a total period of 20 weeks.

The $l.d._{50}$ gradually declined from $10^{7.0}$ at the beginning of the experiment to $10^{2.2}$ after twenty weeks. Table I. The equation $Y = 7.0716 + (-0.3363)X + 0.0058X^2$ best fits these data. Figure 1.

B. Thermolability at 22-25°C.

The virus was titrated at the end of one day and at two day intervals thereafter for a total period of 25 days.

The virus retained its ability to kill all embryos for seventeen days followed by a period of decreased but variable infectivity to the twenty-fifth day when three of ten embryos died. The $l.d._{50}$ declined from $10^{7.0}$ at the beginning of the experiment to $10^{0.7}$ at 23 days and to almost complete inactivation at 25 days. Table II. These data were best fit by the equation $Y = 6.921 + (-0.4336)X + 0.0067X^2$. Figure 2.

TABLE I

THERMOLABILITY OF INFECTIOUS BRONCHITIS VIRUS AT 3-4°C

Time in weeks	log of virus dilutions								$l.d._{50}$
	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
0					5	5	4	3	>7.0
1				5	5	3*	4	3	>6.7
2				5	5	4	3*	1	6.2
4				5	5	5	3	0	6.2
6				5	5	2	1	0	4.8
8				5	3	2	0		4.5
10			5	5	3	1	0		4.2
12	5	5	5	4	2	0			3.6
14	5	5	5	2	0				2.8
16	5	5	5	1	0				2.6
20	5	5	3	1	0				2.2

*deaths per four embryos inoculated

(These footnotes apply to Tables II, III, IV, V, VI, VII, IX, X, XI, XII, XIII, XIV XV, XVI, XVII)

FIGURE 1

THERMOLABILITY OF INFECTIOUS BRONCHITIS VIRUS AT 3-4°C

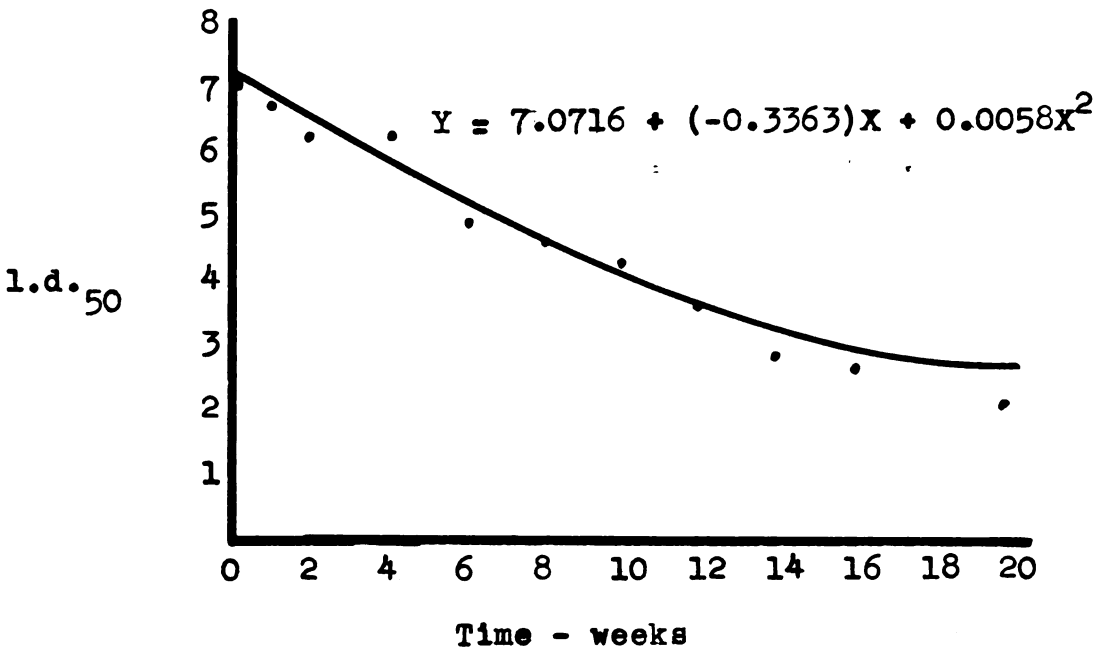


TABLE II

THERMOLABILITY OF INFECTIOUS BRONCHITIS VIRUS AT 22-25°C

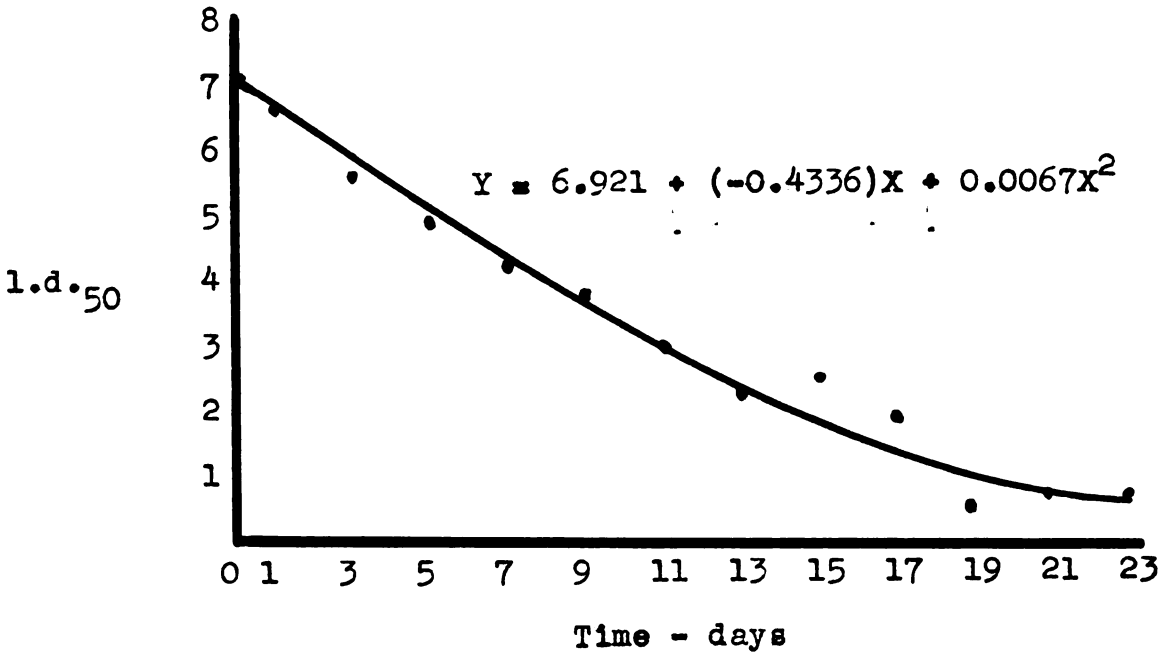
Time in days	Qual. Inf. test#	log of virus dilutions								l.d.$_{50}$
		10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
0	10					5	5	4	3	7.0
1	10					5	5	4	1	6.5
3	10					5	5	1	0	5.6
5	9				5	5	2	0		4.8
7	10			5	5	3	0			4.2
9	9		5	5	5	2	0			3.8
11	10		5	5	2	0	0			2.8
13	8		5	3	0	0				2.2
15	10		4*	4	0	0				2.4
17	10		5	2	0	0				1.8
19	3	3#	2	0	0					0.5
21	7	7#	0	0	0					0.7
23	6##	6##	0	0	0					0.7
25	3	3#	0	0						-

#deaths per ten embryos inoculated
##deaths per nine embryos inoculated

(These footnotes apply to Tables III and IV)

FIGURE 2

THERMOLABILITY OF INFECTIOUS BRONCHITIS VIRUS AT 22-25°C



C. Thermolability at 37°C.

Four hour exposure intervals were used for a period of 60 hours. Qualitative infectivity tests showed that at 36 hours the virus was capable of killing all embryos. From the thirty-sixth to the fifty-sixth hour there was a slight reduction in infectivity, with seven of ten embryos being killed by the virus at 56 hours. Three of ten embryos were killed at 60 hours. The $l.d._{50}$ decreased from $10^{6.8}$ at the start of the experiment to $10^{0.28}$ at 56 hours and to almost complete inactivation at 60 hours. Table III. These data were best fit by the equation $Y = 7.045 + (-0.212)X + 0.0016X^2$. Figure 3.

D. Thermolability at 56°C.

Five minute intervals were used for a total period of 30 minutes.

Qualitative infectivity tests showed that at five minutes after exposure, five of ten embryos were killed by the virus, but at ten minutes the virus was completely inactive. The $l.d._{50}$ decreased from $10^{5.8}$ at the beginning of the experiment to $10^{0.5}$ at five minutes and to zero at ten minutes. Table IV. The equation $Y = 5.8 + (-1.54)X + 0.096X^2$ best fit these data. Figure 4.

E. Comparison of Results.

Summation of the results of the thermolability studies indicate the following rates of inactivation of IBV expressed as temperature/lethal doses/time interval: 4°C/$10^{0.22}$/week, 22-25°C/$10^{0.27}$/24 hours, 37°C/$10^{0.11}$/hour, Figure 5, and 56°C/$10^{1.06}$/minute.

TABLE III

THERMOLABILITY OF INFECTIOUS BRONCHITIS VIRUS AT 37°C

Time in hours	Qual. Inf. test#	log of virus dilutions 10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	$l.d._{50}$
0	10					5	5	4	2*	6.8
4	10					5	4	2	2	6.0
8	10				5	5	2*	0	0	5.0
12	10			5	5	5	0	0		4.5
20	9		5	4	4	2	0	0		3.5
24	9##		5	5	2	4	3			4.5
28	9##		4*	5	0	0	0			2.5
32	10	5	5	2	0	0				1.8
36	10	5	4	2	1	0				1.8
40	8	4	1	0	0					0.5
44	9	4	2	0	0					0.7
48	9	4	0	1	0					0.5
52	8	4	0	1	0					0.5
56	7	7#	0	0	0					0.3
60	3	3#	0	0						-

FIGURE 3

THERMOLABILITY OF INFECTIOUS BRONCHITIS VIRUS AT 37°C

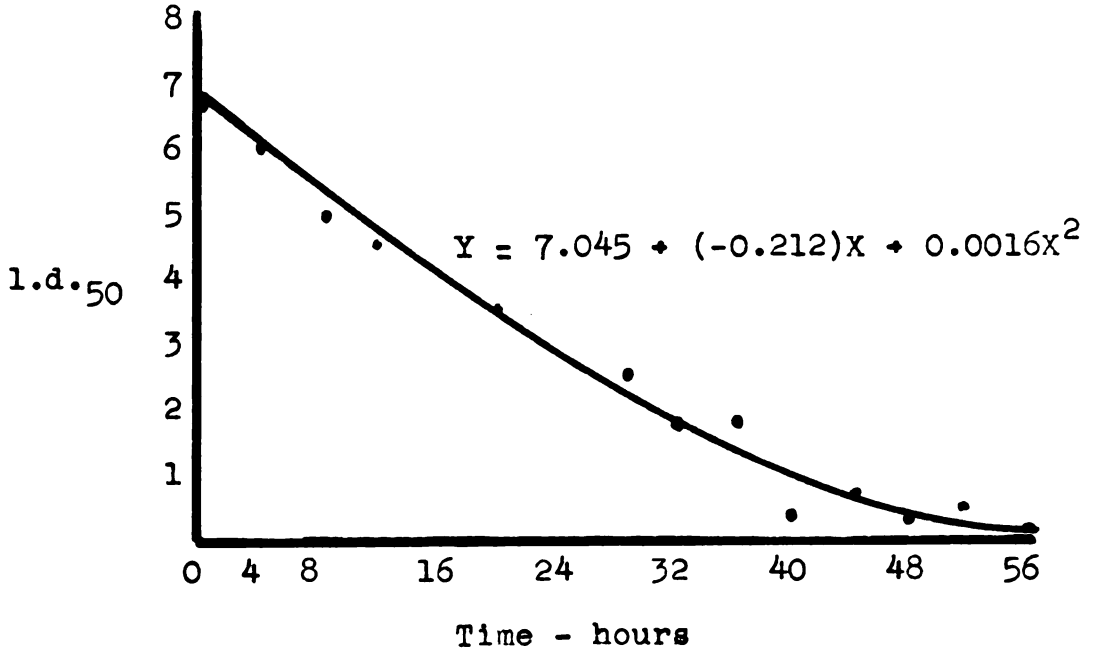


TABLE IV

THERMOLABILITY OF INFECTIOUS BRONCHITIS VIRUS AT 56°C

Time in min.	Qual. Inf. test#	log of virus dilutions 10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	$l.d._{50}$
0	10					5	4	2	1	5.8
5	5	5#	0	0						0.5
10	0									-
15	0									-
20	0									-
25	0									-
30	0									-

FIGURE 4

THERMOLABILITY OF INFECTIOUS BRONCHITIS VIRUS AT 56°C

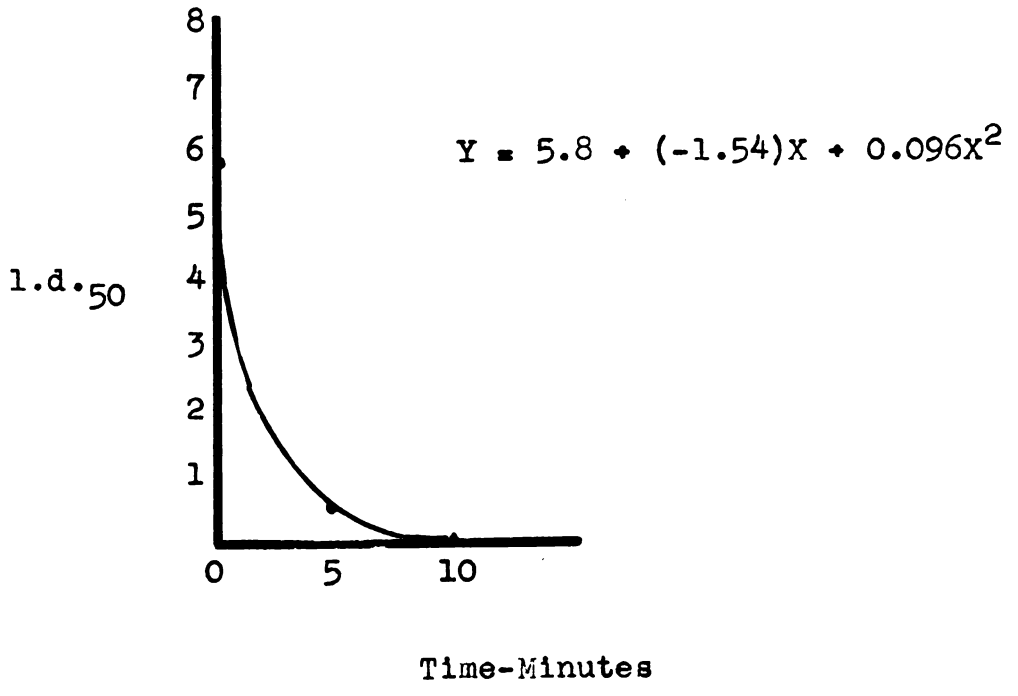
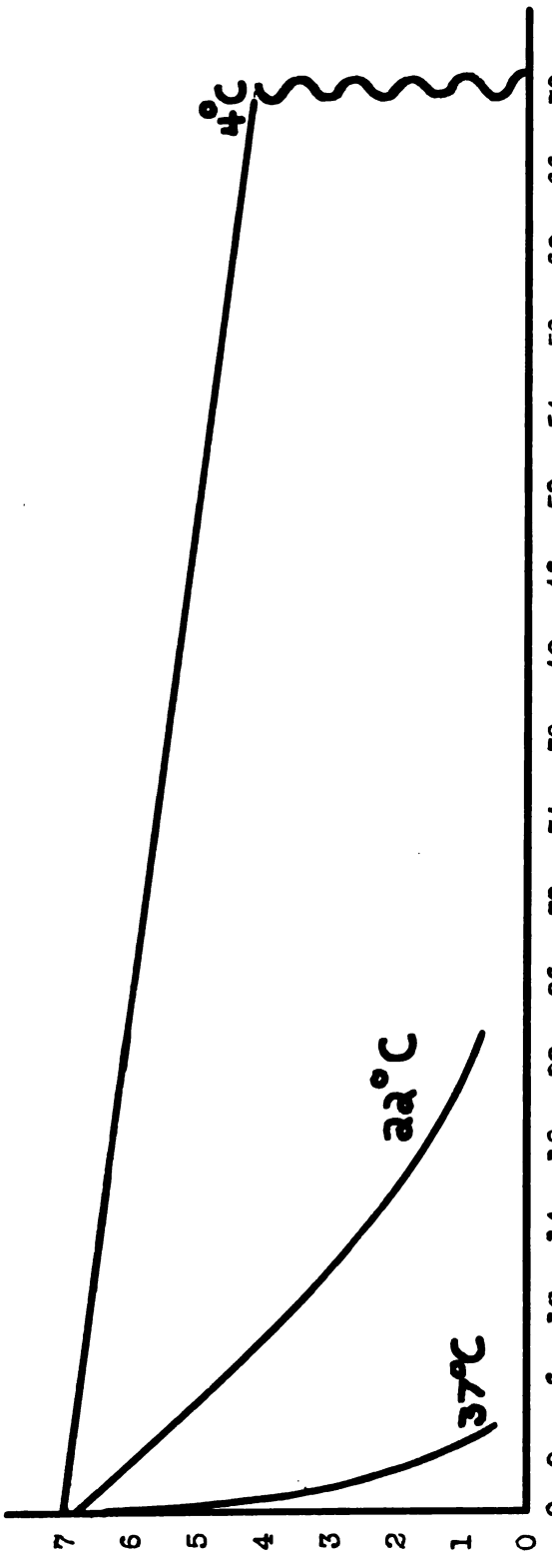


FIGURE 5

IBV THERMOLABILITY AT 4°C, 22 -25°C and 37°C, $Y = a + bX + cX^2$



II. Serum Neutralization Test

A. The Effect of Storage at Different Temperatures on the Neutralizing Capacities of Infectious Bronchitis Immune Serum.

Several workers[14,16,18,53,54,55,65,87] have reported that immune serum will retain its initial neutralizing capacity for as long as one year when stored at 4-6°C. Olitsky and Murphy[69] found that poliomyelitis immune serum had a $l.d._{50}NI$ of $10^{2.3}$ after 20 years storage at 4-5°C. The original titer was not known. Melnick and Ledinko[64] found that neutralizing antibodies against Coxsackie virus were stabile at 65°C for 30 minutes but not at 80°C.

The following experiments were designed to determine the effect of storage of immune serum at 4°C, 22-25°C and 37°C for certain time intervals prior to use in the serum neutralization test. The serum was collected the day before it was subjected to the storage temperature.

Serum was distributed into thin-walled ampoules, 2.0cc per ampoule, heat-sealed and submerged in a thermostatically controlled water bath.

The results indicated that there was no significant change in the neutralizing capacity of IBV immune serum of $l.d._{50}NI$ $10^{6.5}$ following an eight week exposure at 4°C, Table V, or a seven day exposure of IBV immune serum of $l.d._{50}NI$ $10^{4.4}$ at 22-25°C, Table VI. At 37°C, a ten-fold decrease in neutralizing capacity from $l.d._{50}NI$ $10^{6.8}$ to $10^{5.8}$ occurred during a 56 hour exposure. Table VII.

TABLE V

EFFECT OF STORAGE ON INFECTIOUS BRONCHITIS IMMUNE SERUM AT 4°C

	time in weeks	log of virus dilution 10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	ld_{50}	ld_{50}NI
Virus	0					5	5	4	1	>6.5	
SN	0	1	0	0						0.0	>6.5
Virus	2					5	4	4	2	>6.5	
SN	2	1	0	0						0.0	>6.5
Virus	4					5	5	3	1	>6.3	
SN	4	0	0	0						0.0	>6.3
Virus	6					5	5	5	2	>6.8	
SN	6	2	1	0						0.0	>6.8
Virus	8					5	5	4	2	>6.5	
SN	8	2	1*	0						0.0	>6.5

TABLE VI

EFFECT OF STORAGE ON INFECTIOUS BRONCHITIS IMMUNE SERUM AT 22-25°C

	time in days	log of virus dilution								ld_{50}	ld_{50}NI
		10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}		
Virus	0					5	4	2	1	5.8	
SN	0	5	4	0	0					1.4	4.4
Virus	1					5	5	3	2	6.5	
SN	1	5	5	1	0					1.6	4.9
Virus	3					5	5	3	2	6.5	
SN	3	5	4	3	0					2.0	4.5
Virus	5					5	5	2	0	5.8	
SN	5	5	4	1	0					1.5	4.3
Virus	7					5	5	2	0	5.8	
SN	7	5	1	0	0					0.6	5.2

For assessment of the results obtained with the serum neutralization tests of serum stored at 37°C, certain factors must be considered. The virus had an initial titer of $l.d._{50}$ $10^{7.0}$. During the interim between each serum neutralization test the virus was frozen. This resulted in seven cycles of freezing and thawing during the entire experiment. In addition to this effect on the virus, the cumulative exposure of perhaps ten hours at room temperature, for the entire experiment, must be considered. Using the data previously obtained in thermolability studies of the virus at 22-25°C, Table II, Figure 2, exposure of the virus at this temperature for ten hours would result in a calculated $10^{0.2}$ decrease in $l.d._{50}$. The titer of the virus at the termination of the experiment, the fifty-sixth hour test period for the serum, was $10^{6.5}$. Based on previous experience that repeated freezing and thawing was deleterious to the virus, it was assumed that a $10^{0.3}$ decrease could be attributed to this factor. This would mean a total decrease of $10^{0.5}$ as reflected by the $l.d._{50}$ $10^{6.5}$ of the virus at the termination of the experiment.

In order to compensate for these combined effects on the $l.d._{50}$ of the virus at each test period, the initial $l.d._{50}$ may be assumed to decrease by kN, where $k = \Delta\, l.d._{50}/n$, n = total number of freezing cycles, and N = (test period-1).

Example: $k = \frac{10^{0.5}}{7} = 10^{0.07}$

$kN_5 = 10^{0.07} \times 5 = 10^{0.35}$

TABLE VII

EFFECT OF STORAGE ON INFECTIOUS BRONCHITIS

IMMUNE SERUM AT 37°C

	time in hours	log of virus dilution								ld_{50}	ld_{50}NI
		10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}		
Virus	0					5	5	4	3	7.00	
SN	0	3	0	0	0					0.17	6.83
SN	8	3	1	0						0.35	6.58
SN	16	3	1	0						0.35	6.51
SN	24	3	0	1						0.32	6.47
SN	32	3	2	0	0					0.50	6.22
SN	40	3	2	1	0					0.67	5.98
SN	48	2*	1	1	0					0.59	5.99
SN	56	3	2	1	0					0.67	5.83
Virus	56					5	5	4	1	6.50	

At the sixth test period the initial $l.d._{50}$ $10^{7.0}$ for the virus would be considered to have decreased by $10^{0.35}$ to $10^{6.65}$ which was then used in the calculation of the $l.d._{50}$NI at this test period.

B. The Effect of Using Different Numbers of Eggs on Virus Titer and Serum Neutralizing Indices.

In studies of the reproducibility of titrations of PR8 strain of influenza virus, Knight[57] found that with thirty samples the $l.d._{50}$ varied from $10^{12.0}$ to $10^{14.3}$ with $\sigma = \pm 10^{0.132}$, and $v = 10^{0.017}$. von Magnus[85], using ten repeated titrations of influenza PR8, with the $l.d._{50}$ varying from $10^{8.5}$ to $10^{9.1}$, reported $\sigma = \pm 10^{0.23}$.

Knight[57] showed by statistical analysis that when ten embryos per dilution were used, three-fold differences in the concentration of the virus could be readily detected. When five embryos per dilution were used, differences closer than five-fold could not be detected with accuracy.

The purpose of the following experiments was to determine if a significantly greater accuracy of virus titrations and serum neutralization tests could be obtained by using from five to as many as ten eggs per dilution.

1. Procedure and Results

Serum neutralization tests were performed using one, two, and four hour incubation periods at 22-25°C. The virus was titrated only once for this experiment since previous results has shown that there was no significant thermal effect on the virus during a 24 hour exposure period. Table II. Ten eggs were used per dilution. Following inoculation, five eggs per dilution for virus titration as well as for serum neutralization were randomly marked with an X, three with a Y, and two

were left unmarked. The results were then recorded according to the following procedure. For calculations involving ten eggs per dilution, all eggs were counted. For calculations involving eight eggs per dilution, the eggs marked X and Y were used. The eggs marked X and those unmarked were used for calculations involving seven eggs per dilution. For calculations involving five eggs per dilution, one group consisted of those eggs marked with an X, and the other group consisted of those eggs marked with a Y and those unmarked. Table VIII.

With the two virus titrations involving five eggs per dilution, the $l.d._{50}$ differed by $10^{0.2}$ ($10^{6.4}$ and $10^{6.2}$). For practical purposes, the arithmetic average ($\bar{X}$), $10^{6.3}$, was used as a basis for calculation of the $l.d._{50}NI$ when five embryos per dilution were considered. For the entire series of titrations in which five, seven, eight and ten eggs per dilution were considered, $\bar{X} = 10^{6.3}$. From these results, it is evident that for virus titrations using serial ten-fold dilutions, equal accuracy is obtained when five to ten embryos are employed per dilution. From these data, $\sigma = \pm 10^{0.07}$ which compares favorably with those of Knight[57] and von Magnus[85] for titration of influenza virus in embryonating chicken eggs.

For the serum neutralization test in which two groups of five eggs per dilution were involved, the $l.d._{50}NIs$ showed greater differences than those obtained with the virus titra-

TABLE VIII

EFFECT OF NUMBERS OF EGGS ON VIRUS TITRATION

AND SERUM NEUTRALIZATION TEST

A. Virus Titration

Numbers of eggs per dilution	log of virus dilution 10^{-4}	10^{-5}	10^{-6}	10^{-7}	$l.d._{50}$	
10	10	9	7	1	6.3	
8	8	8	6	0	6.3	
7	7	6	5	1	6.3	
5	5	5	4	0	6.4	6.3
5	5	4	3	1	6.2	

B. Serum Neutralizations

Number of eggs per dil.	time in hours	log of virus dilution 10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	$l.d._{50}$		$l.d._{50}NI$	
10	1	10	5	4	0		1.4		4.9	
8	1	6	3	3	0		0.9		5.4	
7	1	6	3	3	0		1.2		5.1	
5	1	5	1	2	0		0.9	1.3	5.5	5.0
5	1	5	4	2	0		1.7		4.5	
10	2	8	4	2	0		0.8		5.5	
8	2	6	3	1	0		0.7		5.6	
7	2	6	4	2	0		1.2		5.1	
5	2	4	1	1	0		0.7	1.0	5.7	5.4
5	2	4	3	1	0		1.2		5.0	
10	4	8	4	2	3	0	1.1		5.2	
8	4	6	3	1	2	0	0.9		5.4	
7	4	6	3	1	2	0	1.1		5.2	
5	4	4	2	0	1	0	0.8	1.1	5.6	5.2
5	4	4	2	2	2	0	1.4		4.8	

tions. For the one hour incubation period, the difference was $10^{0.8}$ ($10^{0.9}$ and $10^{1.7}$), for the two hour period $10^{0.5}$ ($10^{0.7}$ and $10^{1.2}$) and for the four hour period $10^{0.6}$ ($10^{0.8}$ and $10^{1.4}$). The $\bar{X}$, respectively, was $10^{1.3}$, $10^{1.0}$ ($10^{0.95}$) and $10^{1.1}$). The $\bar{X}$ $l.d._{50}NI$ for the entire series of serum neutralization tests was $10^{5.2}$, $\sigma = \pm 10^{0.21}$, and $v = 10^{0.043}$. From these data, it is evident that a significantly greater accuracy was not obtained using more than five eggs per dilution in serum neutralization tests.

C. The Effect on the Serum Neutralization Test of Using Constant Proportions but Different Volumes of Serum and Virus.

It was shown by Bryan and Beard[17] that the amount of purified papilloma virus neutralized by serum depended on the proportions used. In addition, Melnick and Ledinko[64] demonstrated that as the quantity of Coxsackie virus was increased, the quantity of serum required for neutralization was also increased.

The following experiments were performed to determine if there was any effect on the neutralizing capacity of immune serum when different amounts of virus and serum were mixed.

The serum neutralization test was done by mixing 0.5 ml., 1.0 ml., and 2.0 ml., respectively of each virus dilution with an equal amount of immune serum and incubating at room temperature for 45 minutes prior to egg inoculation.

The results, Table IX, show a similar $l.d._{50}$NI for each of the three tests. However, because of the high antibody level, the serum neutralization end-point could not be calculated, and definite conclusions could not be established.

TABLE IX

THE EFFECT ON THE SERUM NEUTRALIZATION TEST OF CONSTANT PROPORTIONS BUT DIFFERENT VOLUMES OF SERUM AND VIRUS

	Vol.	log of virus dilution 10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	ld_{50}	ld_{50}NI
Virus	-					5	5	4	1	6.5	
SN	0.5cc	0	0	0						-	>6.5
SN	1.0cc	0	1*	0						-	>6.5
SN	2.0cc	1	1*	1						-	>6.5

D. The Effect of Amount of Inoculum on the Neutralizing Capacity of Infectious Bronchitis Immune Serum.

Serum neutralization tests were performed using inoculums of 0.05cc, 0.1cc and 0.2cc per embryo for the serum-virus mixtures and 0.1cc for the virus dilutions.

The data indicate that the maximum $l.d._{50}$NI was obtained when equal inoculums were employed for both titrations. In halving or doubling serum-virus inoculums with respect to the virus-dilution inoculum, the serum titer was increased. Table X.

TABLE X

THE EFFECT OF AMOUNT OF INOCULUM ON THE NEUTRALIZING CAPACITY OF INFECTIOUS BRONCHITIS IMMUNE SERUM

		log of virus dilution									
	Inoc.	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	ld_{50}	ld_{50}NI
Virus	0.1					5	5	4	2	6.7	
SN	0.05	2*	2	0	0					0.4	6.3
Virus	o.1					5	5	4	1	6.5	
SN	0.1	1	1*	1						-	>6.5
Virus	0.1					5	5	3	2	6.5	
SN	0.2	3	1	1	0					0.5	6.0

E. The Effect of Dilution and Diluent on the Neutralizing Capacity of Infectious Bronchitis Immune Serum.

By diluting influenza immune serum one in five, Hirst[44] found that a ten-fold decrease in neutralizing capacity occurred. Bryan and Beard[17] reported that neutralization of purified papilloma virus by immune serum varied directly with serum dilution. A ten to one hundred-fold decrease in neutralizing capacity of Newcastle disease immune serum diluted one in five was reported by Brandly et al[15]. A one hundred-fold decrease was observed when the serum was diluted one in ten. Rached[72] reported that dilution of Newcastle disease immune serum one in ten decreased the neutralizing capacity from ten to five hundred-fold. Whitman[86], working with western equine encephalomyelitis virus, reported a decrease in neutralizing titer from $10^{5.2}$ to $10^{2.2}$ when human convalescent serum was diluted ten-fold. Page[70] reported that IB immune serum diluted one in five showed a ten to fifteen-fold decrease in neutralizing capacity. Dilution as high as one in twenty did not appreciably alter the neutralizing capacity beyond that of a one in five dilution.

Bell[13] reported that physiological saline, distilled water and ten per cent normal monkey serum used as diluents had no effect on comparative titrations of poliomyelitis immune serum. With the viruses of poliomyelitis[13,53], Newcastle disease[80], influenza A[50,86], western equine enceph-

alomyelitis[66], pneumonia of mice[51], and myxoma[71], a linear exponential relationship has been found to exist between the serum dilution end-point and the amount of virus neutralized.

With 0.1M phosphate buffer, 0.85 per cent NaCl, and nutrient broth diluents, the $l.d._{50}NI$ ranged for serum undiluted and diluted through 10^{-4} from $10^{5.9}$ to $10^{0.2}$, $10^{5.2}$ to $10^{1.6}$ and $10^{5.4}$ to $10^{1.8}$ respectively. Table XI. From the average $l.d._{50}NI$ of the three diluents at the different concentrations of serum employed, $Y = 5.5 + (-1.11)X$ and $S_y = \pm 0.66$. Figure VI.

TABLE XI

THE EFFECT OF DILUTION AND DILUENT ON THE NEUTRALIZING CAPACITY OF INFECTIOUS BRONCHITIS IMMUNE SERUM

A. Virus titration

log of virus dilution				
10^{-4}	10^{-5}	10^{-6}	10^{-7}	$l.d._{50}$
5	5	4	0	6.4

B. Serum Neutralization

Diluent: Phosphate buffer, 0.1M

Dilution	log of virus dilution									
	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	$l.d_{50}$	$l.d_{50}^{NI}$
10^{0}	5	0	0	0	0				0.5	5.9
10^{-1}		3*	2	1	0				1.8	4.6
10^{-2}		5	3	2	1	0			2.7	3.7
10^{-3}			5	4	4	1	0		4.4	2.0
10^{-4}				5	5	5	3	0	6.2	0.2

Diluent: 0.85 per cent NaCl

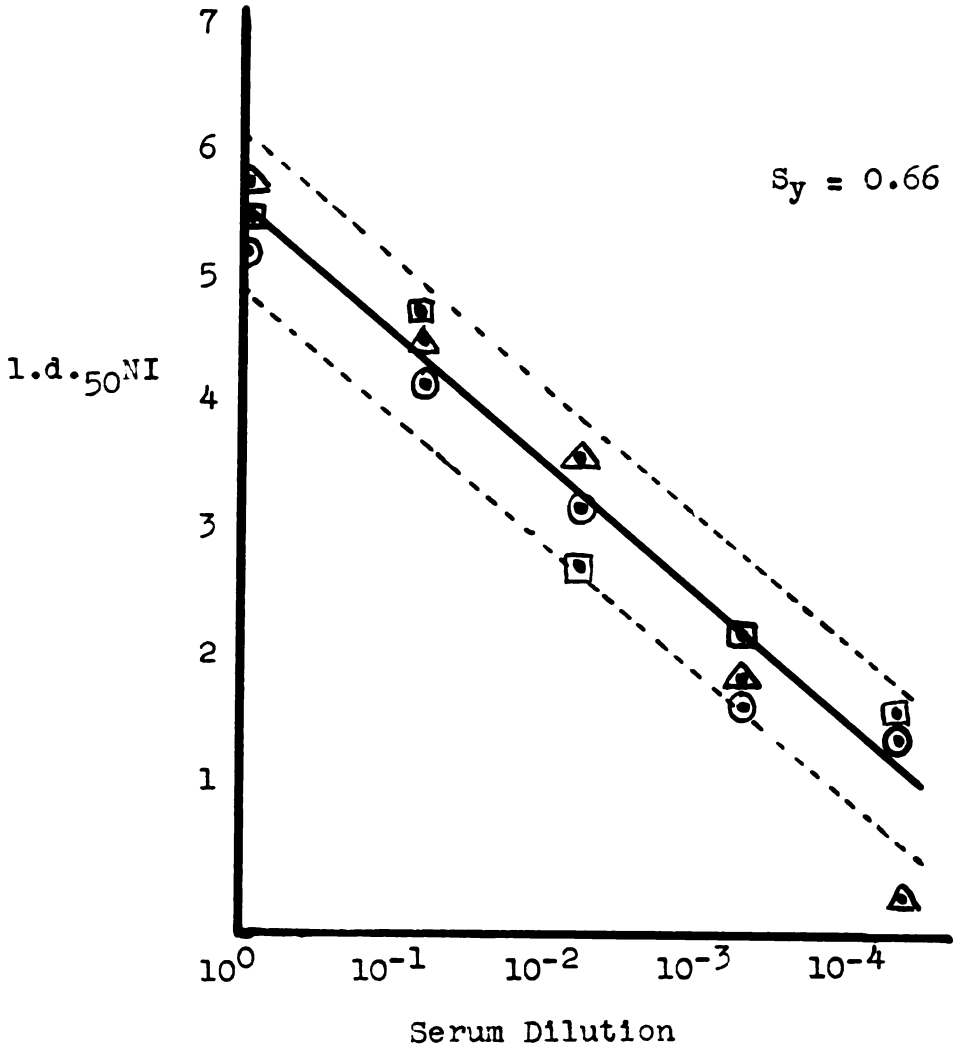
10^{0}	5	2	2	0					1.2	5.2
10^{-1}		5	2	1	1	0			2.3	4.1
10^{-2}			5	2	1	0	0		3.0	3.4
10^{-3}				5	4	0	0	0	4.6	1.8
10^{-4}					5	1	1	0	4.8	1.6

Diluent: Difco nutrient broth

10^{0}	5	2	1	0	0				1.0	5.4
10^{-1}		5	1	0	0	0			1.6	4.8
10^{-2}			5	4	0	0	0		3.6	2.8
10^{-3}				5	3	0	0	0	4.2	2.2
10^{-4}					5	1	0	0	4.6	1.8

FIGURE 6

THE EFFECT OF DILUTION AND DILUENT ON THE NEUTRALIZING CAPACITY OF INFECTIOUS BRONCHITIS IMMUNE SERUM



△ - phosphate buffer
○ - 0.85 per cent NaCl
□ - nutrient broth

F. The Effect of Time and Temperature of Incubation on the Serum Neutralization Test.

Andrewes[2] reported that neutralization of Rous virus by immune serum was more complete when mixtures were incubated at 37°C than at room temperature. However, Olitsky and Casals[68], using poliomyelitis virus, did not find any enhancement of neutralization when the serum-virus mixtures were incubated for two hours at 37°C prior to inoculation, as compared to 15 minutes. Bell[13], using poliomyelitis virus, found that variation in the time and temperature of incubation prior to inoculation produced only slight differences in the end-points.

1. Procedure and Results

Serum neutralization tests were performed at 4°C, 22-25°C and 37°C. In all tests, the ingredients were at thermal equilibrium at the time of mixing and were maintained in water baths at the respective temperatures throughout the incubation period. The tests were made at one, two, four, eight and sixteen hours after incubation at 4°C and 22-25°C. Tables XII, XIII. The virus was titrated only at the first test period. Using data previously obtained, Table I, Figure 1, exposure of the virus at 4°C for sixteen hours would have no significant effect on the titer. However, exposure of the virus to 22-25°C for eight hours would result in a calculated $10^{0.2}$ decrease in $l.d._{50}$, and for sixteen hours almost a $10^{0.4}$ decrease, Table II, Figure 2. This thermal effect on the virus must be considered at 22-25°C.

TABLE XII

EFFECT OF TIME AND TEMPERATURE OF INCUBATION

ON THE SERUM NEUTRALIZATION TEST AT 3 - 4°C

	Incub. in hours	log of virus dilution								ld_{50}	ld_{50}NI
		10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}		
Virus	1					5	5	2	0	5.8	
SN	1	2	2	0	0	0				0.2	5.6
SN	2	4	1	0	0					0.5	5.3
SN	4	4	0	1	0					0.5	5.3
SN	8	2	1	0	1					0.3	5.5
SN	16	2	1	1	0					0.3	5.5

TABLE XIII

EFFECT OF TIME AND TEMPERATURE OF INCUBATION ON THE SERUM NEUTRALIZATION TEST AT 22-25°C

	Incub. in hours	log of virus dilution									
		10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	ld_{50}	ld_{50}NI
Virus	1					5	5	4	0	6.4	
SN	1	4	1	2	0					0.8	5.6
SN	2	4	1	1	0					0.6	5.8
SN	4	4	2	0	1					0.8	5.6
SN	8	2	1	0	0					0.0	6.2
SN	16	2	1	0	0					0.0	6.4

At 37°C, virus titration and serum neutralization tests were made at one, two, four and eight hours after incubation. Table XIV. The virus was titrated at each test period because of its thermolability at this temperature as shown by previous studies. Table III, Figure 3.

The $l.d._{50}NI$ at 4°C varied irregularly from $10^{5.6}$ at the one hour test period to $10^{5.5}$ after sixteen hours. At 22-25°C, the $l.d._{50}NI$ increased slightly but irregularly from $10^{5.8}$ at one hour to $10^{6.1}$ after sixteen hours. At 37°C, the $l.d._{50}NI$ irregularly declined from $10^{5.7}$ at one hour to $10^{5.4}$ after eight hours. The sharp decrease to $10^{4.3}$ which occurred at two hours was considered to be due to technical variations in the test. Disregarding the results for this one test period, all data indicate that prolonged incubation at the various temperatures did not significantly influence the $l.d._{50}NI$. Figure 7.

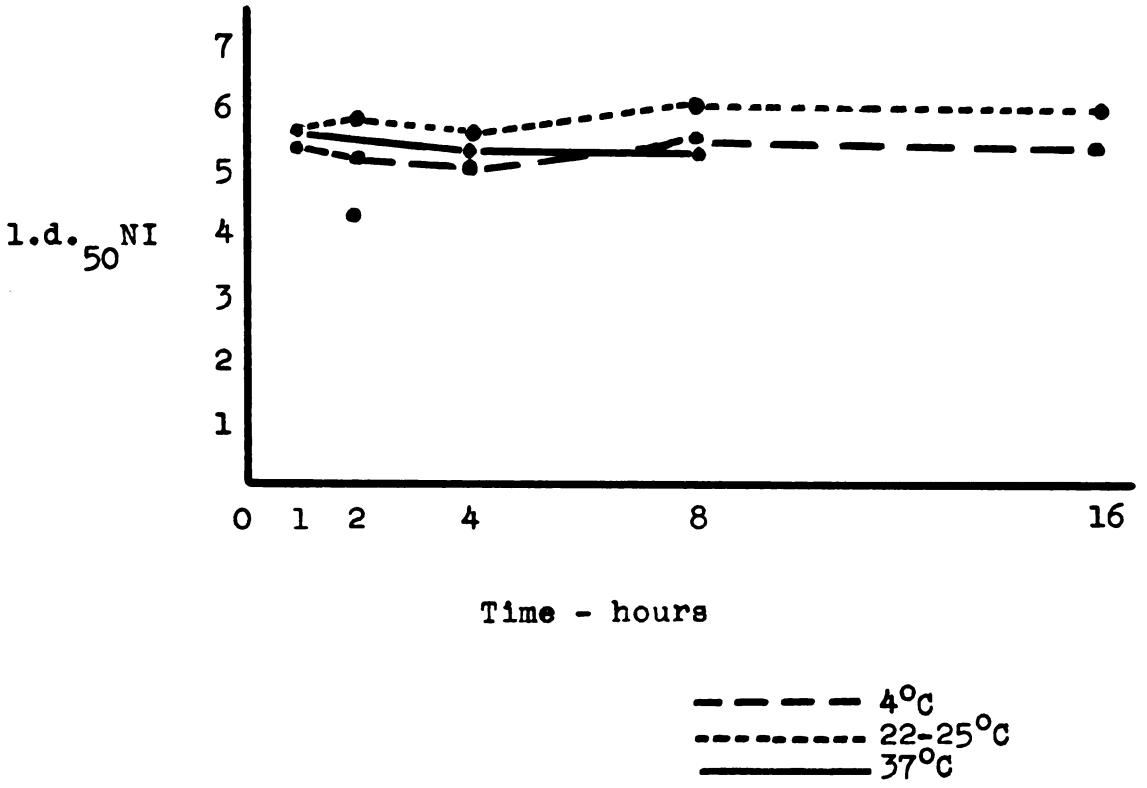
TABLE XIV

EFFECT OF TIME AND TEMPERATURE OF INCUBATION ON THE SERUM NEUTRALIZATION TEST AT 37°C

	Incub. in hours	log of virus dilution								ld_{50}	ld_{50}NI
		10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}		
Virus	1					5	5	4	0	6.4	
SN	1	3	2	1	0					0.7	5.7
Virus	2					5	4	1	0	5.5	
SN	2	4	3	1	0					1.2	4.3
Virus	4					5	4	2	0	5.7	
SN	4	2	1	0	1					0.2	5.5
Virus	8					5	4	0	0	5.4	
SN	8	2	1	0	0					0.0	5.4

FIGURE 7

EFFECT OF TIME AND TEMPERATURE OF INCUBATION ON THE SERUM NEUTRALIZATION TEST AT 4°C, 22-25°C and 37°C



G. The Time-Rate of Virus Neutralization

Bushnell and Erwin[20] found that maximum adsorption of Newcastle disease virus on chicken red blood cells took place within two and one-half to five minutes after mixing the cells and virus. Crawley[21] reported that maximum neutralization of equine encephalomyelitis virus by immune serum was obtained within a few seconds after mixing the virus and the serum.

The following time-rate studies were made to determine the rate of neutralization of infectious bronchitis virus by immune serum.

1. Procedure and Results

Time-rate studies were made at 4°C., 22-25°C, and 37°C in thermostatically controlled water baths. All ingredients were allowed to reach thermal equilibrium prior to combining for the test. Throughout the entire experiment, the virus-serum mixtures were inoculated immediately after preparation and at five minute intervals for a total period of 45 minutes.

The virus was titrated last in all instances to take into consideration any possible deleterious effect of incubation at the various test temperatures.

At 4°C, the $l.d._{50}NI$ increased from $10^{5.3}$ at zero time to an approximate maximum of $10^{6.2}$ after 15 minutes. Accepting $l.d._{50}NI$ $10^{6.2}$ as maximum, 84 per cent of the infectivity neutralizing potential of the serum was utilized at the zero sampling period. Table XV, Figure 8.

TABLE XV

TIME-RATE OF VIRUS NEUTRALIZATION AT 4°C

	time in min.	log of virus dilution								ld_{50}	ld_{50}^{NI}
		10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}		
Virus	55					5	5	5	2	6.8	-
SN	0	5	3	2	0					1.5	5.3
SN	5	5	3	0*	0					1.2	5.6
SN	10	4	3	0						1.0	5.8
SN	15	4	0	2						0.6	6.2
SN	20	4	1*	0						0.5	6.3
SN	25	3	1*	0						0.3	6.5
SN	30	4	1*	0						0.5	6.3
SN	35	5	1	0						0.6	6.2
SN	40	5	0	0						0.5	6.3
SN	45	2	2	0						0.6	6.2

TABLE XVI

TIME-RATE OF VIRUS NEUTRALIZATION AT 22-25°C

	time in min.	log of virus dilution								ld_{50}	ld_{50}NI
		10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}		
virus	55					5	5	2*	2	6.4	-
SN	0	5	3	0						1.2	5.2
SN	5	4	2	2						1.0	5.4
SN	10	5	1	2						0.9	5.5
SN	20	4*	0	0						0.7	5.7
SN	25	5	3*	1**						1.5	4.9
SN	30	4*	2	2*						1.2	5.2
SN	35	4	0	2						0.6	5.8
SN	45	5	0	0						0.5	5.9

**deaths per three embryos inoculated

At 22-25°C, the initial $l.d._{50}$NI increased from $10^{5.2}$ to an approximate maximum of $10^{5.7}$ after twenty minutes of incubation. Table XVI. Figure 8. In this experiment, there was considerable fluctuation in end-points because of the high evidence of non-specific embryo mortality. In addition, results were not obtained at two time intervals due to technical difficulties. However, accepting $l.d._{50}$NI $10^{5.7}$ as the maximum, 91 per cent of the infectivity neutralizing potential of the serum was utilized at the zero sampling period.

The initial $l.d._{50}$NI of $10^{5.9}$ was increased to an approximate maximum of $10^{6.7}$ after ten minutes incubation at 37°C. Accepting $10^{6.7}$ as maximum $l.d._{50}$NI, 86 per cent of the infectivity neutralizing potential of the serum was realized at the zero time sampling period. Table XVII, Figure 8.

These data indicate that between 84 and 91 per cent of the virus neutralizing capacity of immune serum is realized immediately after preparation of the serum-virus mixture. Maximum neutralization occurred 15 and 20 minutes after preparation at the several temperatures employed.

TABLE XVII

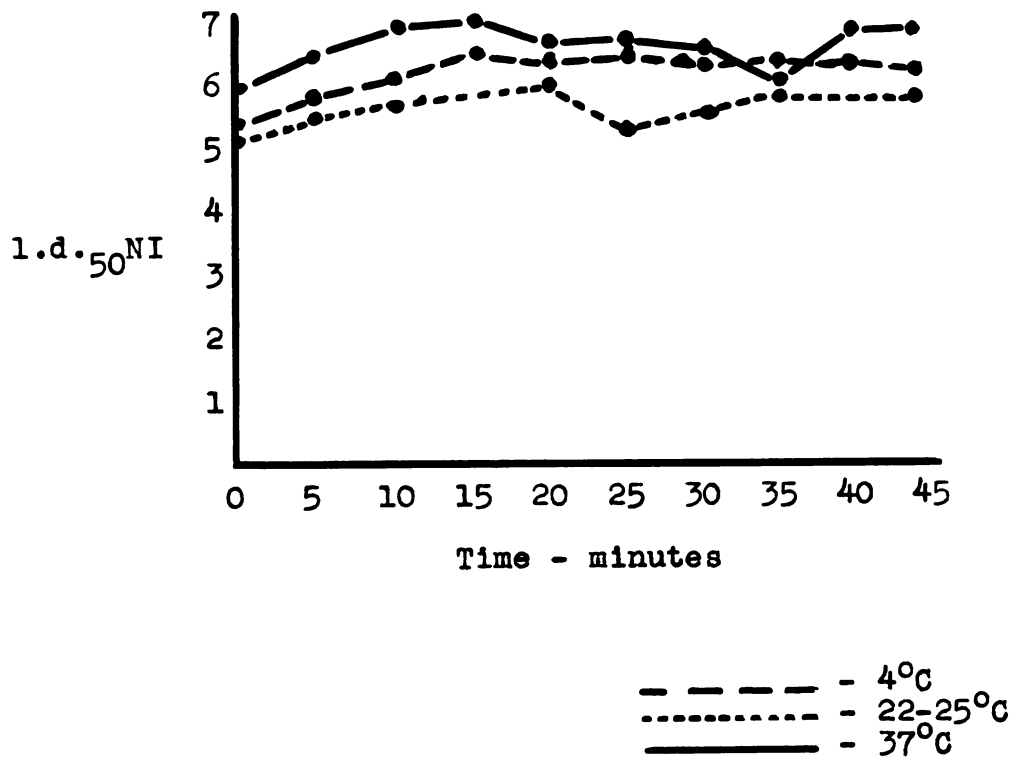
TIME-RATE OF VIRUS NEUTRALIZATION AT 37°C

	time in min.	log of virus dilution								ld_{50}	$ld_{50}NI$
		10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}		
Virus	55					4*	5	4	2	6.7	-
SN	0	2*	3	0						0.8	5.9
SN	5	3*	0	0						0.3	6.4
SN	10	1*	2	0						0.0	6.7
SN	15	1	0	0						-	6.7
SN	20	2*	0	1						0.3	6.4
SN	25	2	0	1						0.0	6.7
SN	30	3	0	0						0.2	6.5
SN	35	4*	1*	1						0.8	5.9
SN	40	2*	0	0						0.0	6.7
SN	45	1*	1	1						0.0	6.7

FIGURE 8

TIME-RATE STUDY AT 4°C,

37°C, AND 25°C.



DISCUSSION

The data obtained from this study of the serum neutralization test for infectious bronchitis of chickens offer certain practical applications for the use of the test as a diagnostic procedure.

Infectious bronchitis virus was more stabile at 4°C than at 22-25°C, 37°C or 56°C for extended periods of time. While the quantitative results did not show marked variation, incubation at 4°C for the serum neutralization test would be more desirable than at higher temperatures in order to minimize any possible deleterious effects of temperature. Serum may be stored at 4°C for as long as eight weeks and at 22-25°C for as long as seven days prior to testing without any change in the neutralizing capacity. At 37°C, a ten-fold decrease in neutralizing capacity occurred after 56 hours storage. These results indicate that 4°C should be routinely employed for storage of serum, but that no deleterious effect would be expected at 22-25°C such as would be experienced in shipping serum to the laboratory from the field.

No advantage in accuracy of the serum neutralization test would be expected when more than five eggs are used per serial ten-fold dilutions for titration and evaluation of the results by the 50 per cent end-point method. This offers a considerable saving in the cost of conducting the test.

The data obtained indicate that inoculation of 0.1 cc of a mixture containing an equal part of serum and virus per egg would be expected to yield the most satisfactory results.

Decimal dilution of serum followed a linear exponential regression equation when either phosphate buffer, 0.85 per cent NaCl or nutrient broth was used as diluents. Either of the diluents could be used with expected replication of results. The $l.d._{50}NI$ for diluted serum could be multiplied by its dilution factor to obtain the titer expected for undiluted serum.

There were no apparent benefits to be derived from prolonged incubation of serum-virus mixtures at 4°C, 22-25°C or 37°C prior to egg inoculation. The results showed that maximum neutralization of the virus by specifically immune serum occurred from 15 to 20 minutes after mixing of the ingredients.

Consideration of these data would indicate that a standard procedure for conducting the serum neutralization test of infectious bronchitis of chickens would consist of using 4°C incubation as a routine measure, although for short periods of time, incubation could be at 22-25°C. Five eggs per dilution, with either phosphate buffer, 0.85 per cent NaCl or nutrient broth as diluent, equal parts of serum and virus, inoculum of 0.1 cc per egg and incubation for about 20 minutes before inoculation of eggs with the serum-virus mixtures should be employed in serum neutralization tests. Virus titrations should be made last to take into consideration any possible deleterious effects on the virus during the incubation period.

SUMMARY

1. IBV was more stabile at 4°C than at 22-25°C, 37°C, and 56°C.

2. IB immune serum undergoes no significant change in neutralizing capacity following exposure of eight weeks at 4°C, seven days at 22-25°C, and at 37°C a ten-fold decrease occurs after 56 hours.

3. No significant increase in virus infectivity was detected by increasing the number of eggs used per dilution from five to seven, eight or ten. The $\bar{X}$ $l.d._{50} = 10^{6.3}$, $\sigma = \pm 10^{0.07}$. For serum neutralization tests using these different number of eggs per dilution, the $\bar{X}$ $l.d._{50}NI = 10^{5.2}$, $\sigma = \pm 10^{0.21}$.

4. Inoculums of 0.1cc of equal parts of serum and virus showed maximum neutralizing capacities.

5. Immune serum can be diluted in phosphate buffer, 0.85 per cent NaCl, or nutrient broth without sacrificing accuracy. Decimal dilutions resulted in a decimal decrease in $l.d._{50}NI \pm 10^{0.66}$.

6. No increase in neutralizing capacity occurs with prolonged incubation from one to eight hours at 37°C, or one to sixteen hours at 4°C and 22-25°C.

7. Eighty-four to ninety-one per cent of the infectivity neutralizing potential of IB immune serum is utilized immediately after serum-virus contact, and maximum neutralization is obtained 15 to 20 minutes later depending upon the temperature of incubation.

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