ANTIBODY RESPONSE OF CHICKENS EXPOSED TO INFECTIOUS BRONCHITIS VIRUS

Thesis for the Degree of M. S.

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Calvin Ames Page

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This is to certify that the

thesis entitled

"Antibody Response of Chickens
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Calvin Ames Page

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W. J. Stapleton
Major professor

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ANTIBODY RESPONSE OF CHICKENS EXPOSED TO INFECTIOUS BRONCHITIS VIRUS

By

Calvin Ames Page

A Thesis

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THESIS

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HISTORICAL REVIEW

Infectious Bronchitis of Chickens

Infectious bronchitis was first studied by Schalk and Hawn[37] in 1931 in North Dakota as a respiratory disease of chicks. In 1933 Bushnell and Brandly[9] reported the disease in Kansas and Gibbs[22] reported it in Massachusetts. Since that time the disease has been found to be widely distributed throughout the country.[3] The disease has been recently reported in England[1] and in Holland.[38]

Infectious bronchitis was originally thought to be confined to chicks, but it is now recognized as an important disease of chickens of all ages.[16] The morbidity rate in chicks is usually high and the mortality rate may be as high as 90 per cent of those infected.[37] In adult chickens the mortality rate is negligible, but in a laying flock there is a temporary cessation of egg production which may persist for several weeks.[2,16]

The etiological agent of the disease is a virus capable of passing through all grades of Berkefeld[2,4,16] and Seitz[16] filters.

Electron microscopy has shown the virus to be round with a mean diameter of 90 mu. Filamentous projections may be present on some virus particles.[35]

Affected chicks exhibit symptoms of sneezing, gasping, tracheal rales, depression and coarse chirping.[2,9,37] In adult chickens the symptoms are less severe than those in

chicks.[16,18] The incubation period is from 3 to 7 days and the duration of the disease is usually from 14 to 21 days.[5] According to Hofstad,[25] the outstanding histopathologic alterations of infectious bronchitis are a thickening of the tracheal mucous membrane and submucosa due primarily to edema and diffuse, leucocytic infiltration. Inclusion bodies were not observed. Significant changes were not seen in the liver, spleen and kidney. Gross lesions included mucus accumulations in the lower trachea and bronchi, congestion and edema of the lungs and cloudiness of the air sac membranes. Facial swellings may be observed in young chicks.[2,4,16,25,37]

The virus is found most abundantly in tracheal exudates and in the lungs although Bushnell and Brandly[9] reported successful transmission of the disease with specimens of blood, spleen, liver, and kidney from infected chickens. Beach and Schalm[2] demonstrated that the disease could be regularly transmitted to healthy chicks by intranasal or intratracheal injection of tracheal exudate containing the virus. Delaplane and Stuart[16] reported successful transmission of the disease by subcutaneous and intraperitoneal injections of the virus as well as injections into the thymus gland and the air spaces of the bones. Komarov and Beaudette[31] stated that some chickens recovered from the disease may be carriers and serve as potential reservoirs of infection. Hofstad[26,28] demonstrated that recovered chickens could transmit the

virus as long as 35 days after recovery. Fabricant[21] was able to isolate the virus in chickens up to 21 days following exposure. Levine and Hofstad[32] demonstrated that the virus can be air-borne for a distance of at least 5 feet and that ultra-violet irradiation was ineffective in control of the spread of the disease.

Chickens recovered from infectious bronchitis are immune to subsequent natural or artificial exposure to the virus.[16] Exposure of chickens 6 to 8 weeks of age with chicken-propagated virus has been employed in an immunization program in the New England area. Protection against the disease was attained in chickens during the egg-laying period by employing this procedure.[5,18,19] This program has been extensively used with considerable success in highly congested poultry areas but has not been recommended in areas in which the disease is not widespread.[19]

Neutralizing antibodies can be detected in serum of recovered chickens with <u>in vitro</u> serological tests.[5,26,30] Jungherr and Terrell[30] demonstrated by serum neutralization tests a naturally acquired passive immunity in chicks hatched from eggs laid by hens recovered from the disease. The egg yolk was the principal medium for the transference of neutralizing antibodies. In yolk pools collected on the 11th, 12th, and 16th days of incubation there was an average of 9,583, 8,000 and 990 neutralizing doses respectively. In pooled serums of chicks 1,2,3 and 4 weeks after

hatching there were 1,000, 875, 10 and 3 neutralizing doses respectively. Random samples collected between the 5th and the 17th week failed to show demonstrable antibodies.

Hofstad and Kenzy[29] confirmed the naturally acquired passive immunity as reported by Jungherr and Terrell,[30] but they were also able to produce infectious bronchitis by injection of the virus into 4,6,7 and 10 day-old chicks hatched from eggs laid by immune parent stock. These findings would indicate that naturally acquired passive immunity might not be effective in completely protecting chickens against infectious bronchitis during the first 10 days after hatching.

Cultivation of Virus in Embryonating Chicken Eggs

Cultivation of the virus in embryonating chicken eggs was first reported by Beaudette and Hudson.[4] The first passage of the virus via the chorioallantoic membrane produced little observable effect on the embryo. After 6 to 8 passages in eggs the virus became lethal to the extent that a few of the embryos were killed. Similar observations were made by Delaplane and Stuart[16,17] who reported that with succeeding transfers via the chorioallantoic membrane the virus became progressively more virulent to the embryo and less virulent to the chicken. At about the 65th passage the virus was completely adapted to the embryo as evidenced by mortality of all inoculated embryos. The virus at

this stage of adaptation was slightly virulent for chickens. After the 90th passage the virus was completely non-infective to chickens and was incapable of stimulating the production of antibodies.

Delaplane[20] reported that the virus could be more rapidly adapted to the embryo by inoculation via the allantoic cavity than via the chorioallantoic membrane. Evidence of this adaptation was the noticeable dwarfing of the embryo on the 1st passage of the virus. Similar results were reported by Fabricant[21] who considered that dwarfing and curling of embryos was pathognomonic of infection with the virus. Loomis[33] substantiated the findings of Delaplane[20] and Fabricant[21] that dwarfing and curling of embryos during the first 8 passages of virus isolated from chickens was pathognomonic of infection with the virus. Microscopic alterations of the embryo as reported by Loomis[33] consisted of pneumonia, hepatic necrosis, interstitial nephritis and congestion in the spleen. The chorioallantoic membrane and the amnionic membrane were edematous.

<u>In vitro</u> serum-neutralization tests have been used as a diagnostic aid. The potentialities of the test were first discussed by Van Roekel.[3,39]

In studies of the distribution of egg-adapted strain V114D of the virus in embryos inoculated via the allantoic cavity, Cunningham and El Dardiry[13] found the greatest concentration of the virus in the

chorioallantoic membrane, followed in order by the allantoic fluid, amnionic fluid and liver. The yolk material was inocuous. The concentration of the virus was greater in materials harvested from living embryos than from dead embryos at the same postinoculation period. There was no advantage in the use of a 10^{-3} dilution of virus as inoculum as compared to undiluted virus-infected allantoic fluid. Cunningham and Stuart[11] reported that an inoculum of 0.05cc. per egg via the allantoic cavity resulted in a higher concentration of the virus in the allantoic fluid than when 0.1cc. and 0.2cc. inoculums were employed. Groupe[23] demonstrated an interference phenomenon associated with the virus harvested from infected embryos maintained at normal incubation temperatures for 24 hours after death. Heat-inactivated virus-infected allantoic fluid containing the interfering material, if injected 30 minutes before injection of the active infectious bronchitis virus, delayed the rate of death of the embryos. This phenomenon was not demonstrated in the allantoic fluid of normal embryos, living infected embryos and infected embryos dead less than 2 hours when treated in a similar manner.

Cunningham and Stuart[10] found that the following chemical agents usually employed for disinfection were capable of inactivating the virus in 3 minutes or less; phenol 3% and 1%; liquor cresolis saponatus, 3% and 1%;

sodium hydroxide 1-20; potassium permanganate, 1-1,000 and 1-10,000 and mercuric chloride, 1-1,000.

The pH stability of the virus at 4°C was studied by Cunningham and Sturt.[12] The virus was more stable in an acid medium for the first 60 days, but from the 60th to the 170th day there was a shift to a greater stability in an alkaline medium. The virus remained active in allantoic fluid at pH 7.80 for 100 days and for 100 days and for 170 days in a phosphate buffer at pH 7.79.

Diagnosis

The criteria usually employed for diagnosis of infectious bronchitis are the clinical history, symptoms and lesions,[2,9,16,18,25] isolation of the virus with the production of characteristic pathologic alterations of embryonating chicken eggs,[20,21,23] and serologic tests.[3,39] The virus does not agglutinate chicken red cells. This is of value for differentiation of infectious bronchitis virus and Newcastle disease virus as the Newcastle virus will agglutinate red blood cells.[27]

Experimental Procedures

The object of these experiments was to study the antibody response of chickens at certain time intervals following exposure to infectious bronchitis virus as a possible aid in the diagnosis of the disease by serum neutralization tests.

Two different strains of infectious bronchitis virus V114D and VR Lot 277 were used. Strain V114D was adapted to cultivation in embryonating chicken eggs and was capable of killing all embryos in 48 hours after inoculation via the allantoic cavity. This strain was used as the antigen in the serum neutralization tests. VR Lot 277 was a chicken-propagated strain of the virus and had not been cultivated in embryonating chicken eggs. This strain was supplied through the courtesy of Dr. Henry Von Roekel, Department of Veterinary Science, University of Massachusetts, Amherst, and was used for exposure of the chickens. All chickens were supplied through the courtesy of the United States Department of Agriculture Regional Poultry Laboratory, East Lansing, Michigan and had been raised in complete isolation under an unusually rigid quarantine.

VR Lot 277 was received as a saline suspension of tracheal washings from infected chickens. Four 3-week-old chicks were inoculated with 0.4cc. of the suspension via the intranasal and intratracheal routes. Forty-eight hours following inoculation the chicks displayed typical

symptoms of infectious bronchitis. The trachea and lungs were collected, pooled, and ground with sand in a mortar and pestle and suspended in 10 ml. of nutrient broth. (Difco). The tissue suspension was centrifuged at 2,500 r.p.m. for ten minutes and the supernatant fluid was transferred with a pipette into a sterile vial. The fluid was then treated with penicillin and streptomycin, 10,000 units each per ml. of suspension, and six 10-day embryonating chicken eggs were inoculated with 0.1cc. of the fluid via the allantoic cavity. The inoculum produced characteristic dwarfing and curling in the embryos by the 3rd post-inoculation day.

In vitro serum neutralization test[14]

Ten-day embryonating chicken eggs were used in the neutralization tests. These eggs had been incubated in an electric, forced-draft incubator at 99-99.5°F (86-88°F wet bulb thermometer). The site for injection via the allantoic cavity was determined by trans-illumination of the egg and selection of an area free of large blood vessels about 3 mm. below the base of the air cell. A small hole was drilled through the shell, without piercing the shell membrane, by means of a small drill attached to the chuck of an electric motor. Another hole was drilled above the air cell to serve as an air vent to allow equalization of the pressure produced by injection of the inoculum into the egg. The holes were painted with tincture of metaphen and the shell membrane above the air cell

was pierced with a sterile teasing needle.

Serial ten-fold dilutions of V114D virus-infected allantoic fluid were prepared in sterile nutrient broth (Difco) in the proportions of 0.7 ml. of virus to 6.3 ml. of diluent. Serum-virus mixtures were prepared in separate tubes by mixing equal parts of each virus dilution and undiluted test serum. It was necessary to dilute some serums 1 in 10 with sterile 0.85 per cent NaCl to give enough volume to conduct the test. These mixtures were incubated at room temperature (about 22-25°C) for 45 minutes. Quantitative determinations of the virus dilution with sterile nutrient broth. In all mixtures, 0.5 ml. of the virus dilutions were mixed with 0.5 ml. of the undiluted serum, diluted serum or nutrient broth.

Five eggs were used per dilution and each egg received an inoculum of 0.1cc. Inoculations were made with a 1.0 cc. B-D Yale tuberculin syringe fitted with a 27 gauge, ½ inch needle. The eggs for the virus titration were inoculated last to make allowances for any possible deleterious effect of incubation on the virus. After inoculation the holes in the shells were sealed with melted paraffin and the eggs were reincubated and candled daily for 5 days. Death of the embryos during the first 24 hours was attributed to non-specific causes and these embryos were not included in the final results.

The results of the serum and virus titrations were evaluated according to the 50 per cent end-point formula

of Reed and Muench[36] expressed as LD_{50}.

The difference between the reciprocal of the virus titer and the reciprocal of the serum titer was considered to be the neutralization index (NI). The antilog of the NI represented the numbers of neutralizing doses (ND). The NI of the serums diluted 1 in 10 was considered to be 10 times the difference between the virus titer and the serum titer.

Exposure of Chickens

Eight 196-day old Single-Comb White Leghorn cockerels were used in the experiments. Six chickens were exposed to strain VR Lot 277 and two chickens were maintained as controls at the Regional Poultry Laboratory.

On January 25, 1950, blood was collected by cardiac puncture from all chickens for determination of pre-exposure neutralizing indices. At intervals of 1,2,3,4,6,8, 10 and 12 weeks after exposure, 20 ml. of blood were collected from each chicken. The blood samples were slanted, the serums collected after about 24 hours and bacterial sterility tests were made. In a few instances the serums were contaminated with bacteria. These samples were passed through a Swinney filter. All serums used in the neutralization tests were bacteriologically sterile.

On January 25, 1950 the six experimental chickens were exposed to strain VR Lot 277, as follows: Chickens K1318B2, K1462Y3 and K1507V3 were inoculated with 0.1 cc. of the virus suspension via the intranasal route and with

0.1 cc. via the intratracheal route. Chickens K1460Z3, K1457S4 and K1516F2 were inoculated with 0.2 cc. of the virus suspension via the intranasal route and with 0.2 cc. via the intratracheal route. On the 3rd day after exposure symptoms of infectious bronchitis were first observed. These symptoms persisted for 5 days and were accompanied by a loss of appetite.

Table 1 -- Chicken K1402F2

Control

Serum	Virus Titer*	Virus dilutions 10^{-1}**	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Serum Titer*	NI+	ND++
Pre-exp.	5.16	5	4≠	5	3	2	4.68	.46	3
1 week	5.63			5	5	3	5.16	.47	3
2 weeks	5.63			5	5	2	4.83	.80	6
3 weeks	4.68			5	5	1	4.63	.05	1
4 weeks	5.83			5	5	5	≥5.00	.83	≥7
6 weeks	6.00			5	5	3	≥5.00	.40	≥3
8 weeks	4.63			5	4	1	4.50	.13	2
10 weeks	6.00			5	5	1	4.63	1.37	23
12 weeks	4.75			5	2	1	4.50	.25	2

These footnotes apply to Tables 1 through 11.
*Reciprocal of negative exponent of logarithm base 10.
**Embryos dead out of 5 inoculated per dilution.
+Neutralization index.
++Neutralizing doses.
≠One embryo of 5 inoculated died due to trauma.
‡Serum diluted 1 in 10.

Table 2 -- Chicken K1463T3

Control

Serum	Virus Titer*	Virus dilutions 10^{-1}**	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Serum Titer*	NI+	ND++
Pre-exp.	5.16	5	5	5	1	0	3.63	1.53	34
1 week	5.63			5	4≠	2≠	5.00	.63	4
2 weeks	5.63			5	5	2	4.83	.80	6
3 weeks	4.68			5	2	0	3.83	.85	7
4 weeks	5.83			5	5	3	≥5.00	.83	≥7
6 weeks	6.00			5	5	1	4.63	1.37	23
8 weeks	4.63			5	2	0	3.83	.80	6
10 weeks	6.00			5	4	1	4.50	1.50	32
12 weeks	4.75			5	3	1	4.31	.44	3

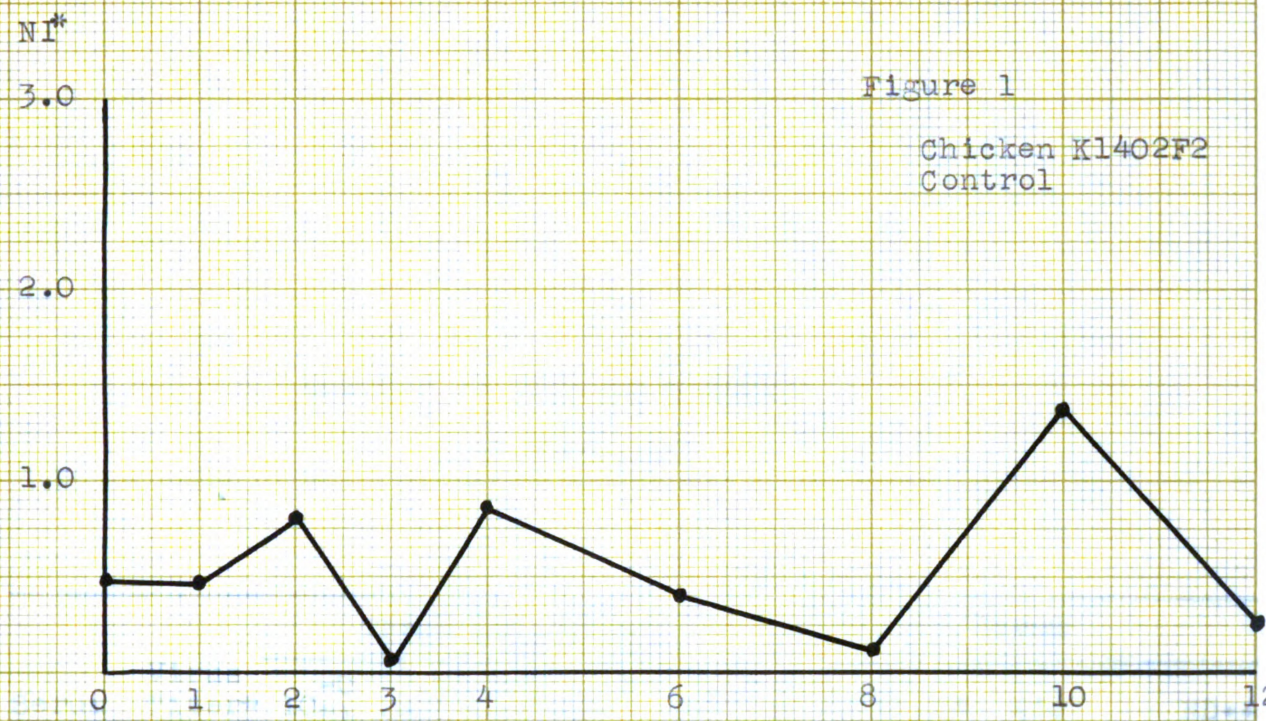


Figure 1

Chicken K1402F2
Control

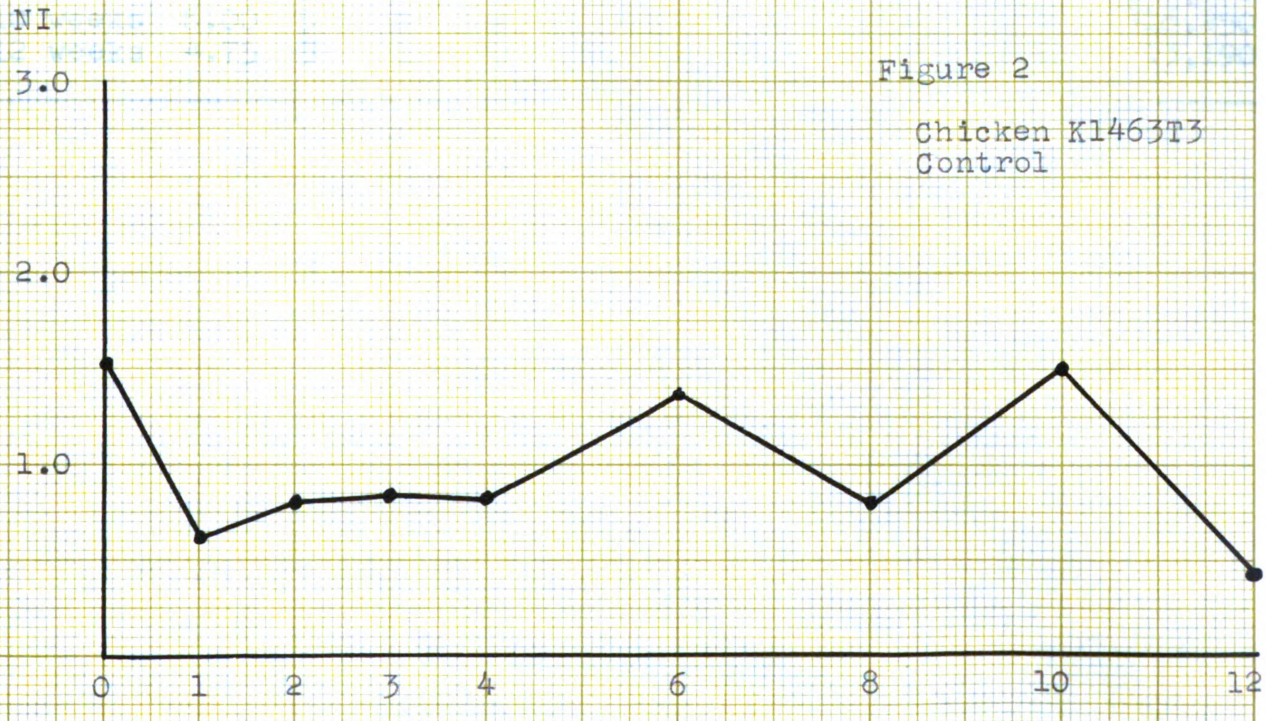


Figure 2

Chicken K1463T3
Control

This footnote applies to Figures 1 through 10.
*NI expressed as the exponent of Log 10

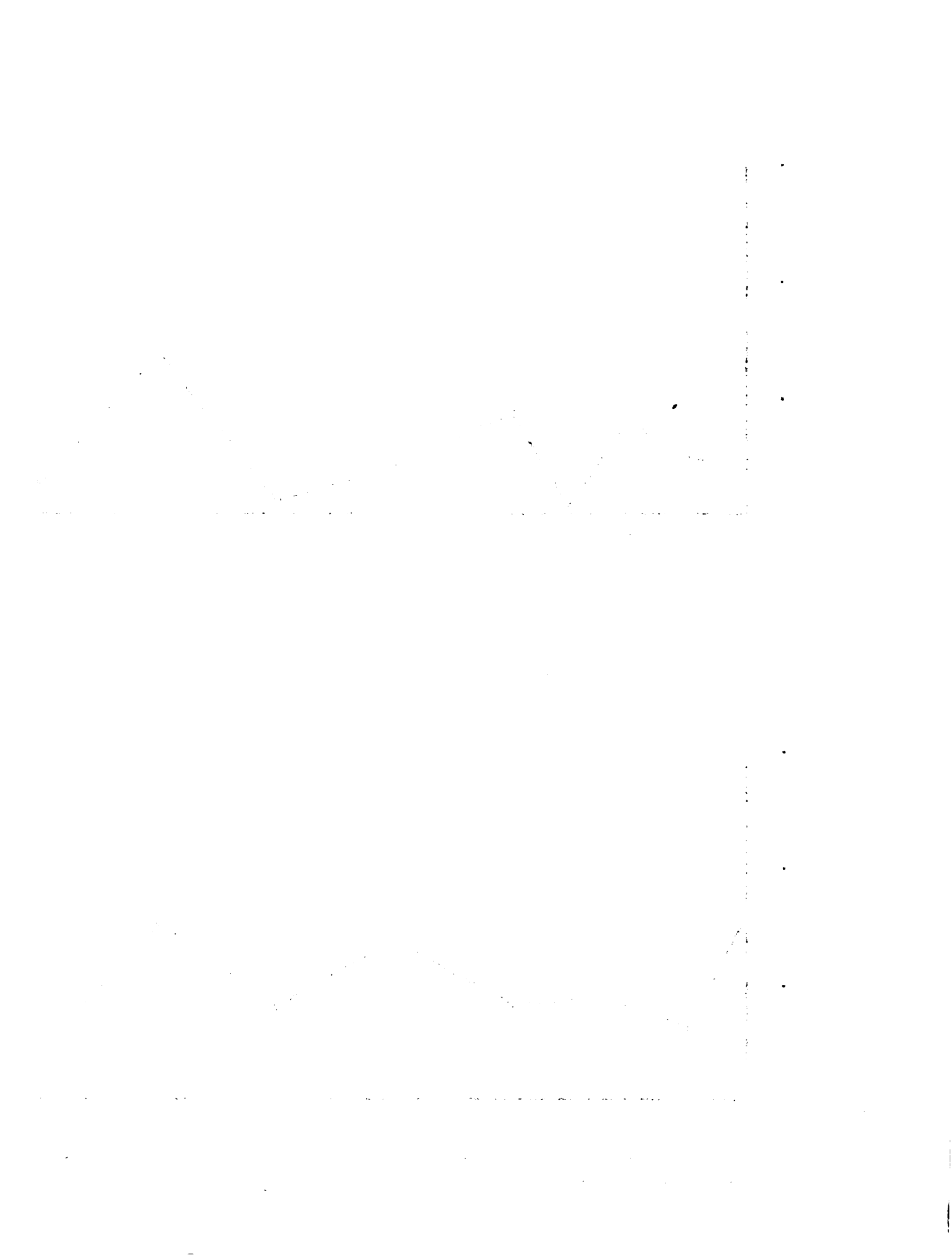


Table 3 -- Chicken K1460Z3

Experimental

Serum	Virus Titer*	Virus dilutions 10^{-0}**	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Serum Titer*	NI+	ND++
Pre-exp.	5.16		4	5	5	2	2	4.22	.94	9
1 week	5.63	5	5	5	4	4	0	4.25	1.38	24
2 weeks	5.17		5	4	3	0	0	3.00	2.17	148
3 weeks	4.68	5	4	1	0	1	0	1.63	3.05	1,122
4 weeks	5.83	5	2	1				1.00	4.83	67,610
6 weeks	6.00	4	1	0				0.50	5.50	316,250
8 weeks	6.00	5	2	0	0	0	0	0.83	5.17	147,900
10 weeks	6.00	5	0	0				0.50	5.50	316,250
12 weeks	4.75	5	1	0	0			0.63	4.12	13,190

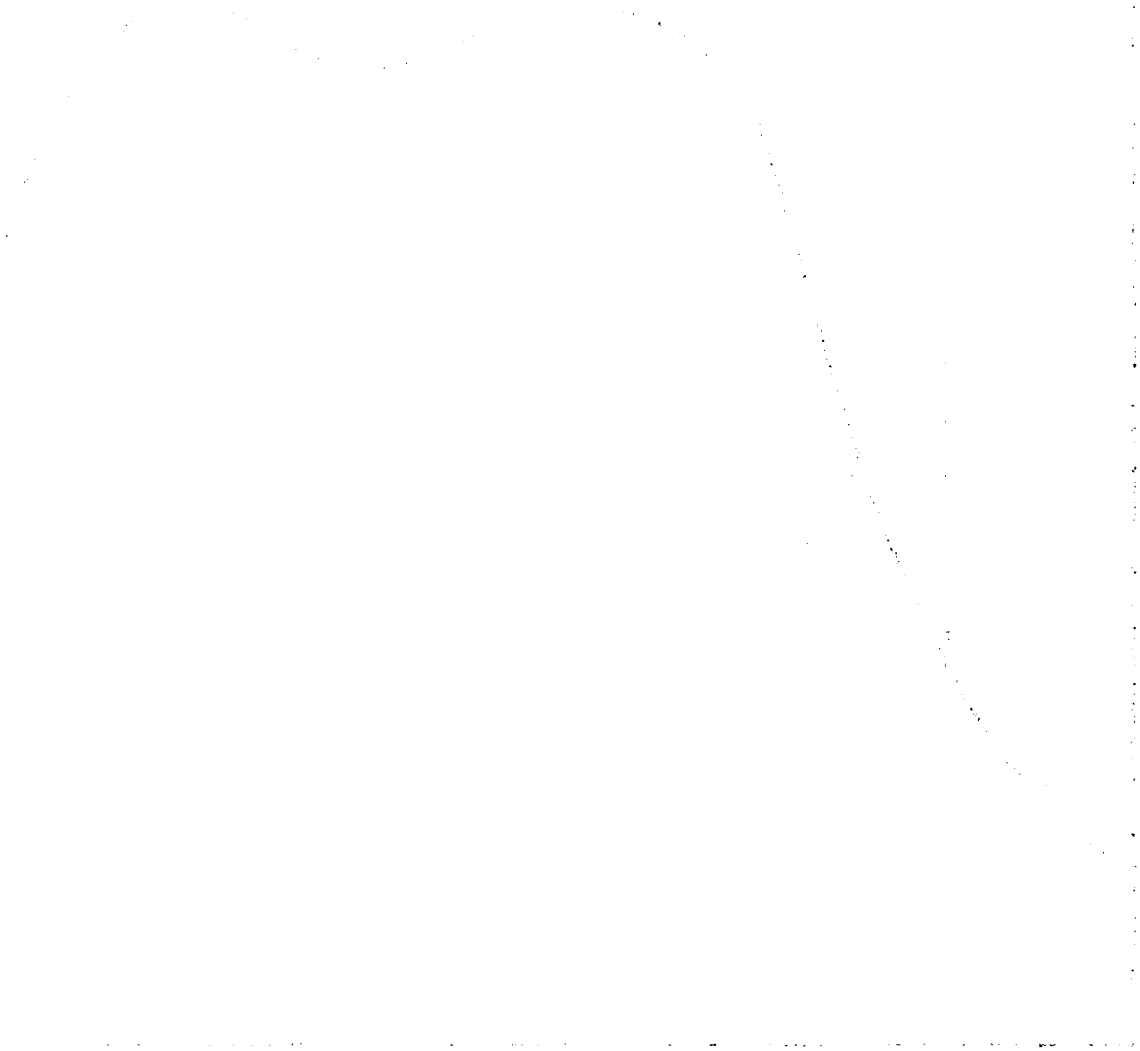


Table 4 -- Chicken K1318B2

Experimental

Serum	Virus Titer*	Virus dilutions 10^{-0}**	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Serum Titer*	NI+	ND++
Pre-exp.	5.16		5	4	5	3	1	4.32	.84	7
1 week	5.63	5	5	5	5	4	1	4.50	1.13	13
2 weeks#	5.17	5	5	5	5	3	0	4.17	2.00	100
3 weeks	4.68	5	3	0				1.17	3.51	3,236
4 weeks	5.83	4	2	2				1.00	4.83	67,610
6 weeks	6.00	5	0	0				0.50	5.50	316,250
8 weeks	4.75	5	3	0				1.17	3.58	3,820
10 weeks#	4.75	5	1	0	0			0.63	5.12	131,900
12 weeks	4.75	2	1	0	0			0.50	4.25	17,780

Table 5 -- Chicken K1457S4

Experimental

Serum	Virus Titer*	Virus dilutions 10^{-0} **	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Serum Titer*	NI+	ND++
Pre-exp.	5.16		5	5	5	1	2	3.88	1.28	19
1 week	5.53	5	5	4≠	4	4	0	4.25	1.38	24
2 weeks#	5.17	5	5	3	2	0	0	2.50	3.67	4,680
3 weeks	4.68	3	2	0				0.50	4.18	15,135

Chicken died Feb. 21, 1950. Cause of death peritonitis.

Table 6 -- Chicken K1462Y3

Experimental

Serum	Virus Titer*	Virus dilutions 10^{-0} **	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Serum Titer*	NI+	ND++
Pre-exp.	5.16		5	5	5	4	1≠	4.53	.63	4
1 week	5.63	5	5	4≠	4	4	1	4.38	1.25	18
2 weeks#	5.17	5	4≠	5	5	2	0	3.83	2.34	220

Chicken died Feb. 8, 1950. Cause of death ruptured artery.

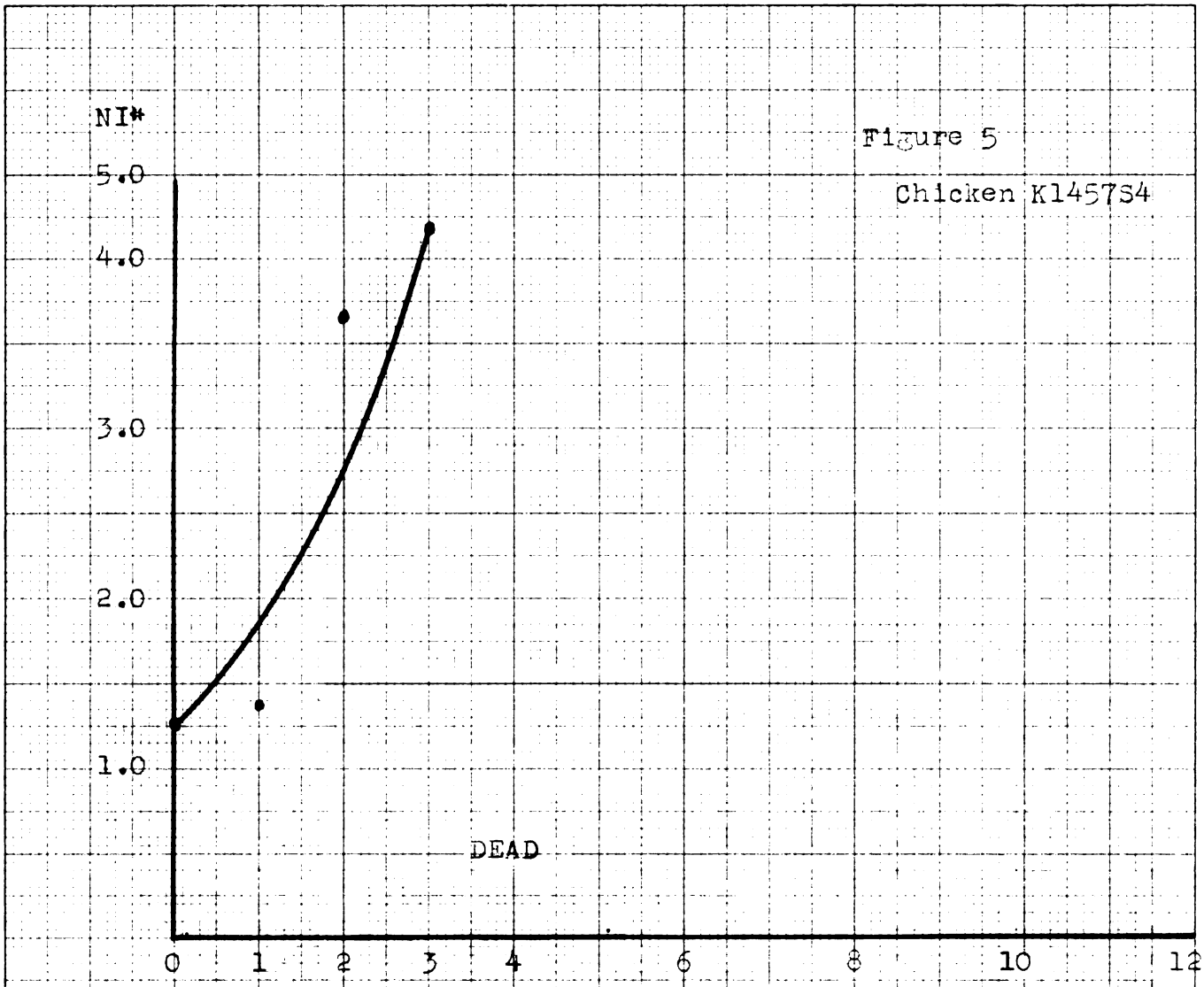


Figure 5

Chicken K1457S4

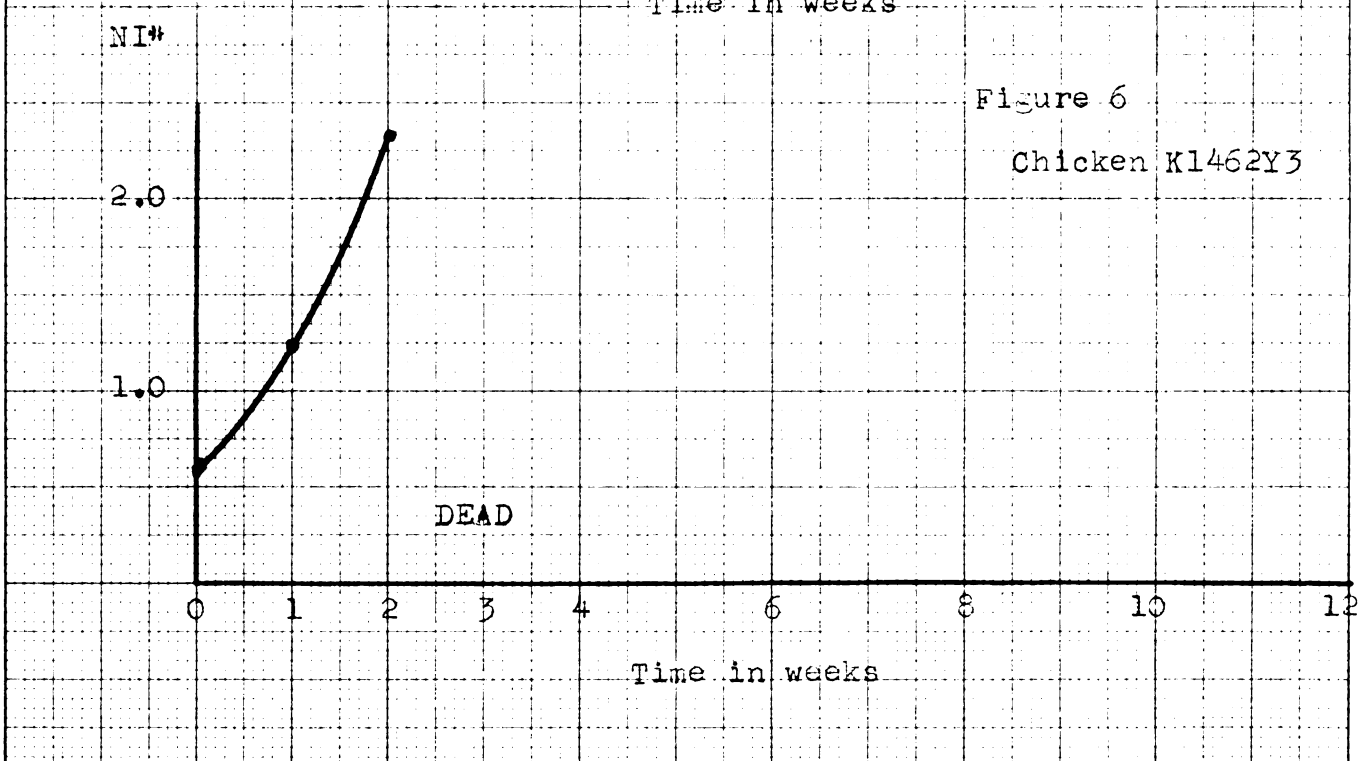


Figure 6

Chicken K1462Y3

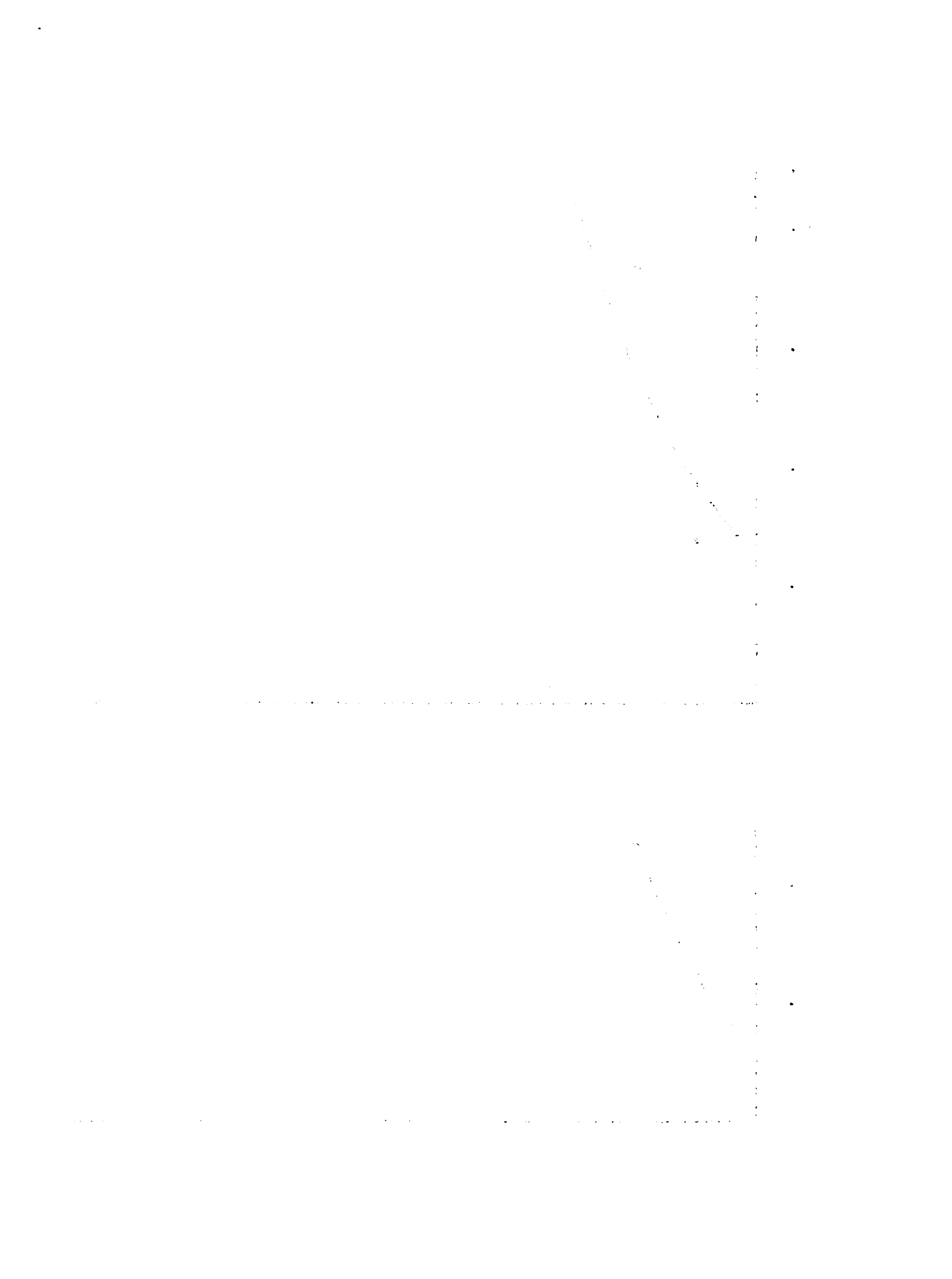


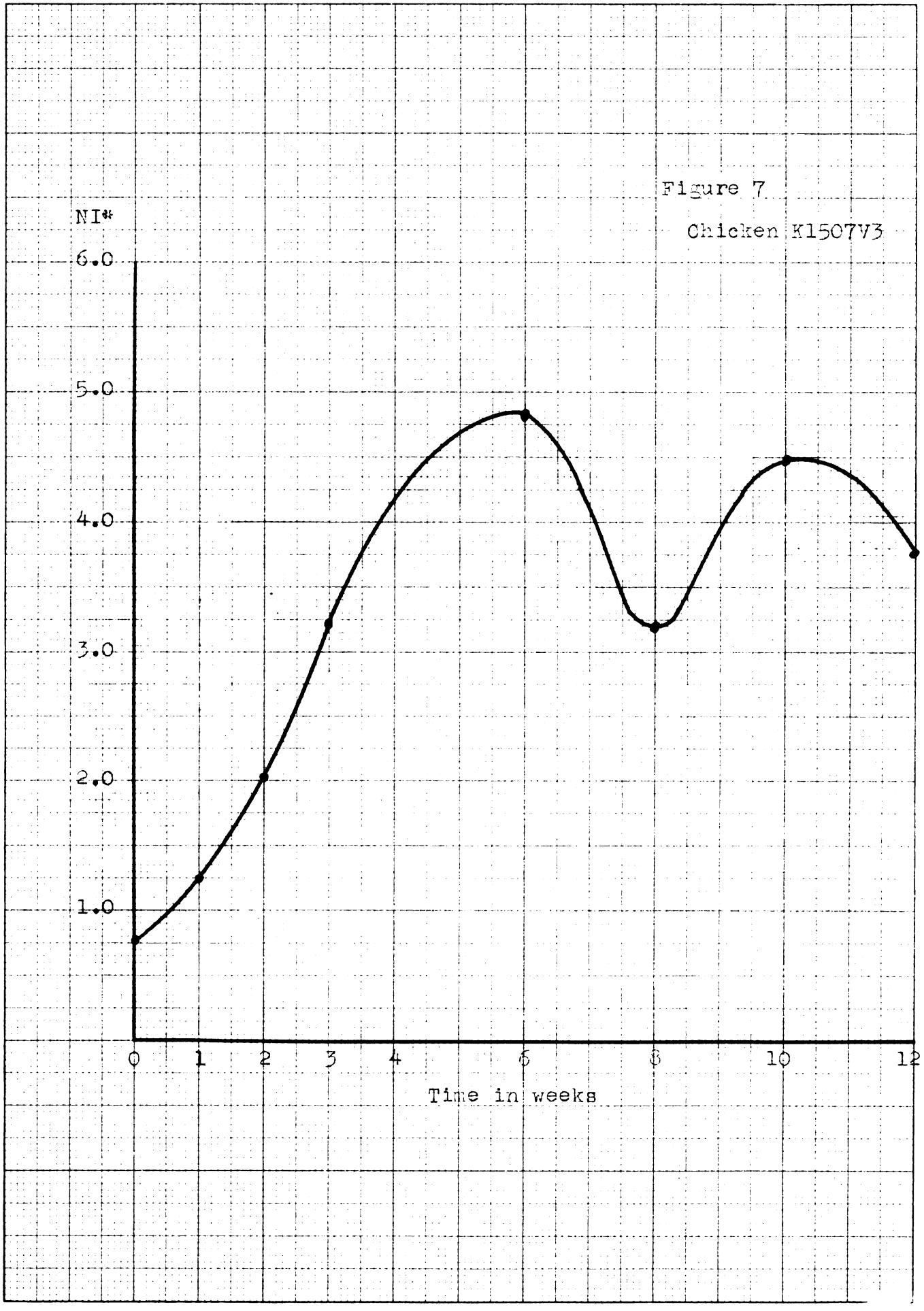
Table 7 -- Chicken K1507V3

Experimental

Serum	Virus Titer*	Virus dilutions 10^{-0}**	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Serum Titer*	NI+	MD++
Pre-exp.	5.16		5	5	5	4	0	4.38	.78	6
1 week	5.63	5	5	5	5	4	0	4.38	1.25	18
2 weeks	5.22			3≠	2≠	1	1	3.20	2.02	105
3 weeks	4.68	5	4	0	1			1.48	3.20	1,585
4 weeks	Insufficient amount of serum									
6 weeks	6.00	5	3	0	0			1.17	4.83	67,620
8 weeks	4.61	5	4	0	0			1.38	3.23	1,778
10 weeks	6.00	5	5	0	0	0		1.50	4.50	31,625
12 weeks	4.75	5	2	0	1	0		1.00	3.75	5,623

Figure 7

Chicken K1507V3



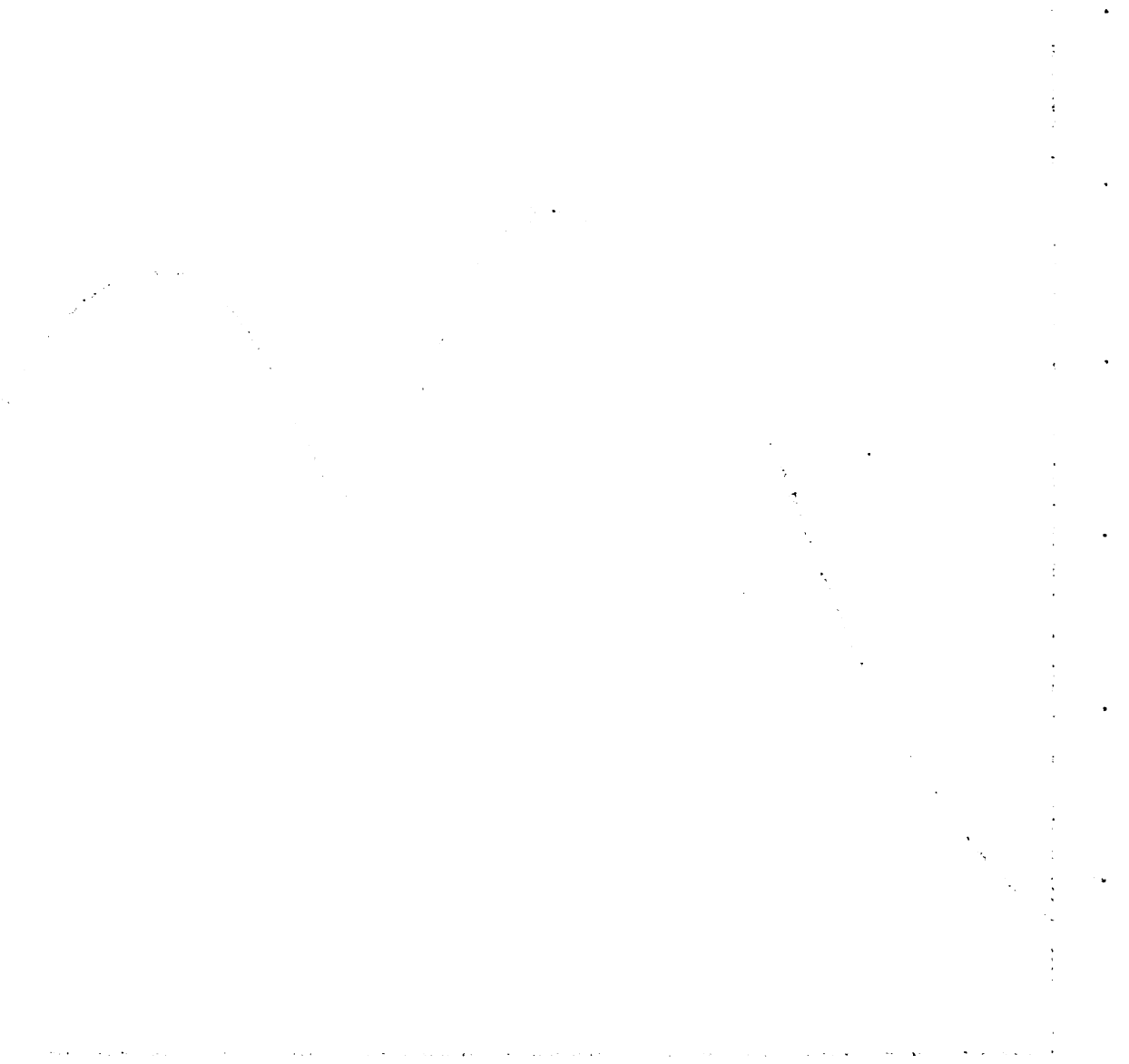


Table 8 -- Chicken K1516F2

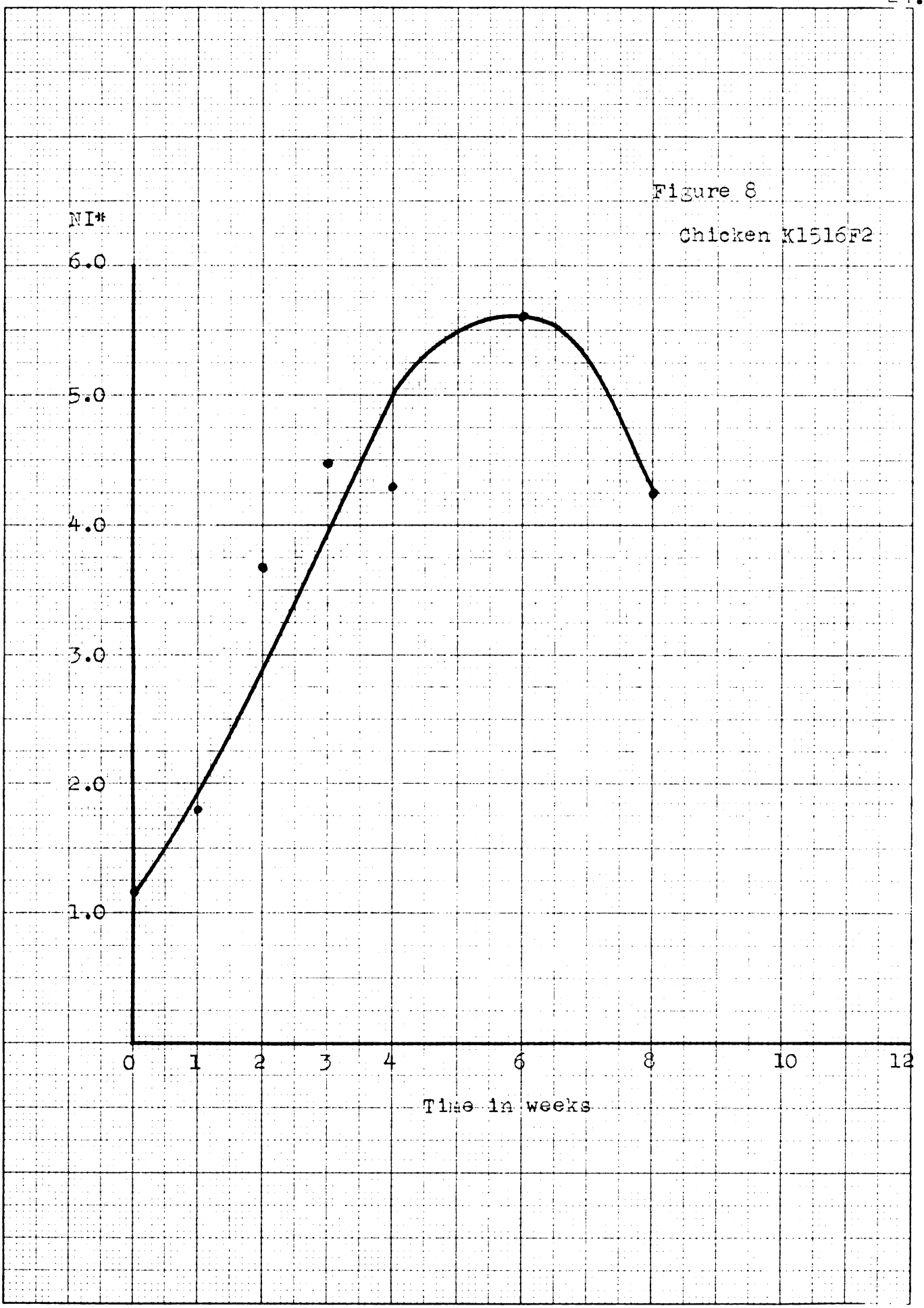
Experimental

Serum	Virus Titer*	Virus dilutions 10^{-0}**	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Serum Titer*	NI+	ND++
Pre-exp.	5.16		5	5	5	2	1	4.00	1.16	14
1 week	5.63	4	5	5	5	2	0	3.83	1.80	63
2 weeks	6.00	5	5	3	0	0	1	2.31	3.69	4,898
3 weeks	5.17	4	2	0	0	0	0	0.68	4.49	30,906
4 weeks	4.63	3	1	0	0	0		0.32	4.31	20,415
6 weeks	6.00	4	0	0	0			0.38	5.62	416,870
8 weeks	4.75	5	0	0				0.50	4.25	17,780

Chicken died March 29, 1950. Cause of death unknown.

Figure 8

Chicken K1516F2



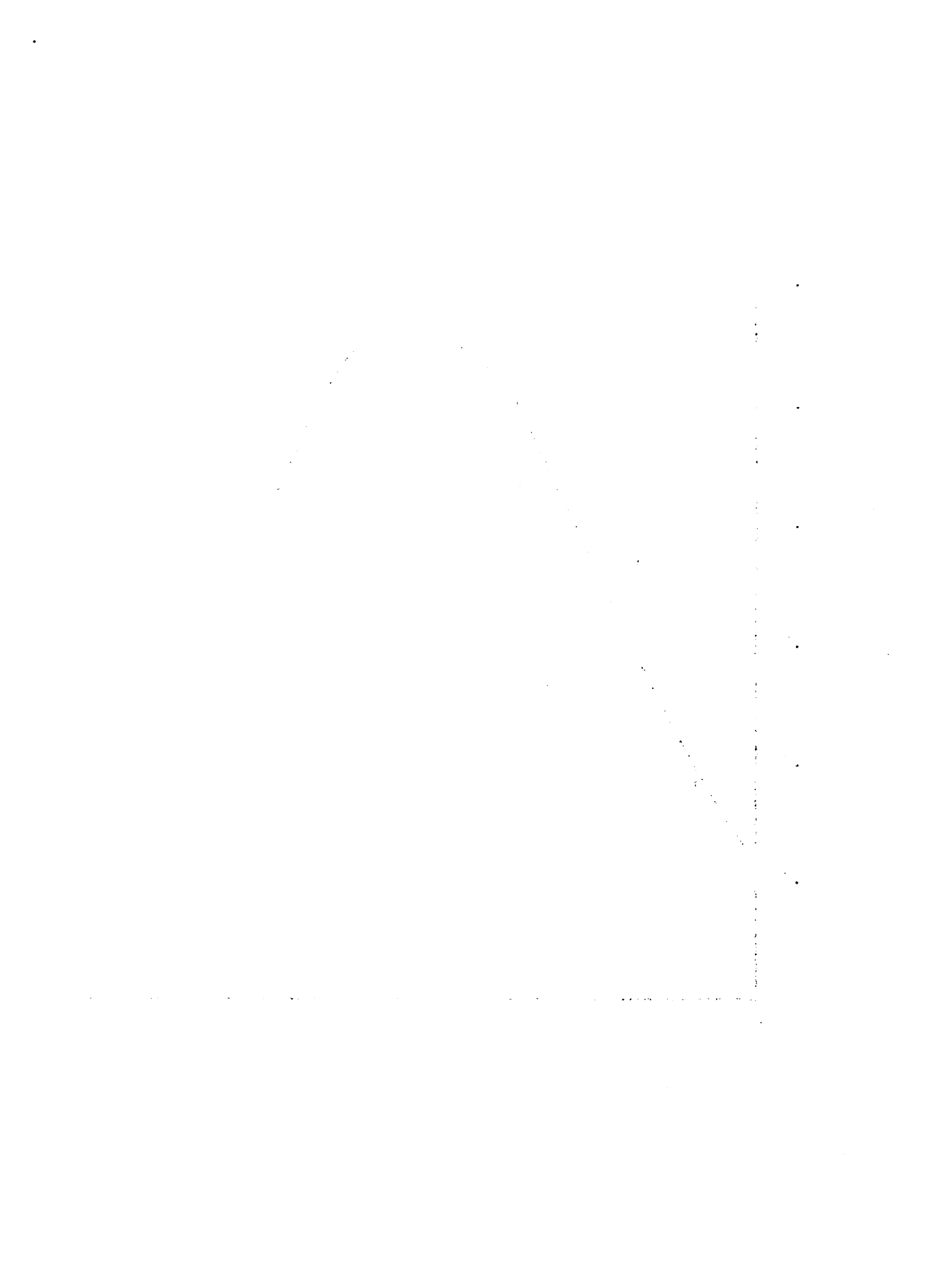


Table 9 -- Summation of neutralization indices and averages for the experimental chickens

	Neutralization indices						
Serum	K1460T3	K1318B2	K1457S4	K1462Y3	K1507V3	K1516F2	Average
Pre-exp.	0.94	0.84	1.28	0.63	0.78	1.16	0.94
1 week	1.38	1.13	1.38	1.25	1.25	1.80	1.37
2 weeks	2.17	2.00	3.67	2.34	2.02	3.69	2.65
3 weeks	3.05	3.51	4.18	--	3.20	4.49	3.69
4 weeks	4.83	4.83	--	--	--	4.31	4.66
6 weeks	5.50	5.50	--	--	4.83	5.62	5.36
8 weeks	5.17	3.58	--	--	3.23	4.25	4.06
10 weeks	5.50	5.12	--	--	4.50	--	5.04
12 weeks	4.12	4.25	--	--	3.75	--	4.04

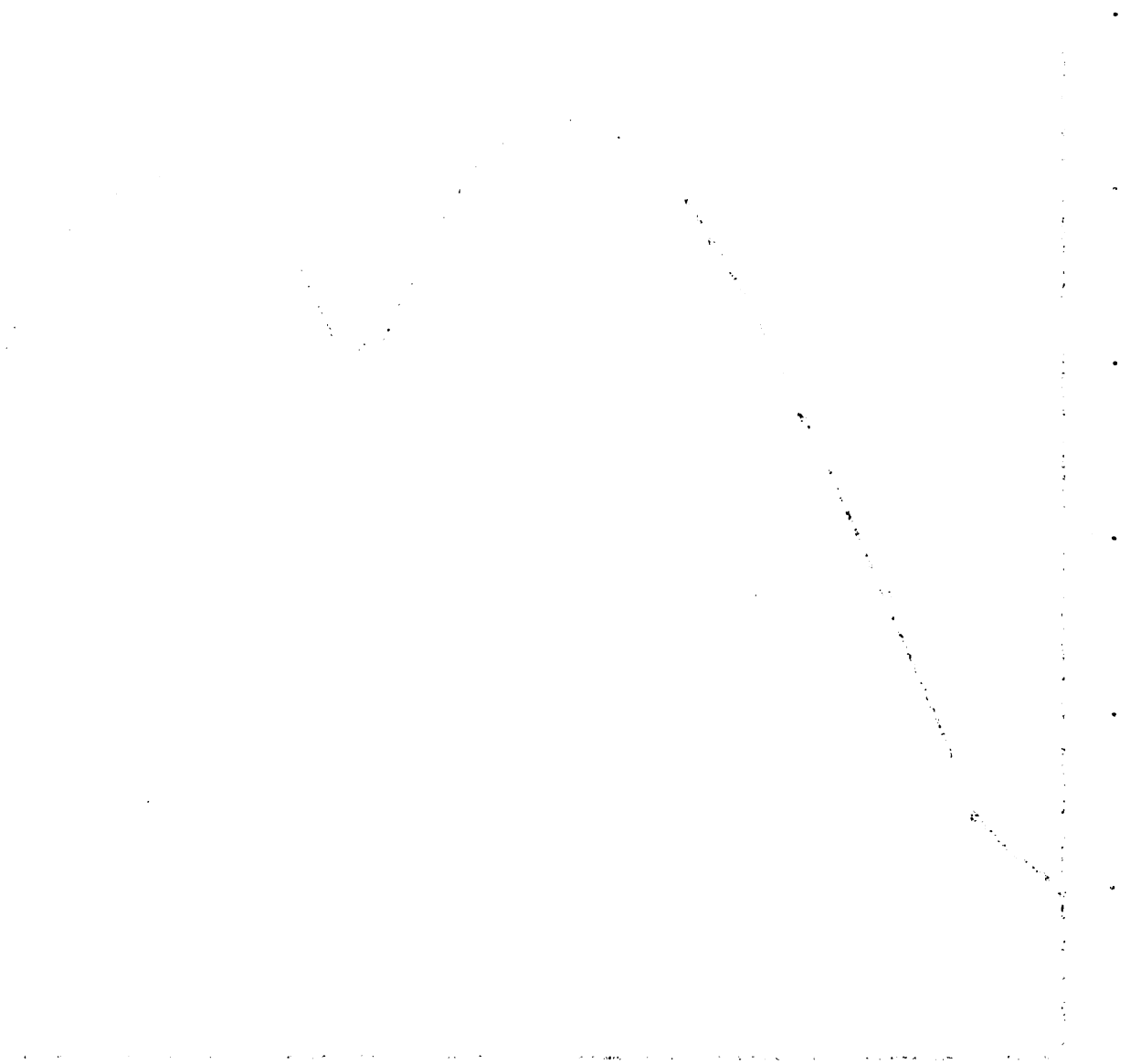


Table 10 -- Neutralizing indices of pooled serums

Pooled Serum Sample	Virus Titer*	Virus dilutions						Serum		
		10^{-0}**	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Titer*	NI+	ND++
K1402F2	5.32				4	5	3	5.17	.15	2
K1463T3	5.32				5	5	4	5.32	.00	0
1 week	5.32				5	0	0	3.50	1.82	66
2 weeks	5.32			0	1	0	1	0.70	4.62	41,690
3 weeks	5.32		2	1	0	0	0	0.50	4.82	66,070
4 weeks	5.32	5	2	2	1	0	1	1.63	3.69	4,892
6 weeks	5.32	5	3	0	0			1.17	4.15	14,130
8 weeks	5.32	5	1	0	0			0.63	4.69	48,920
10 weeks	5.32	5	2	0	0			0.83	4.49	30,910
12 weeks	5.32	3	1	0	0	0		0.32	5.00	100,000

Figure 10

Pooled serums

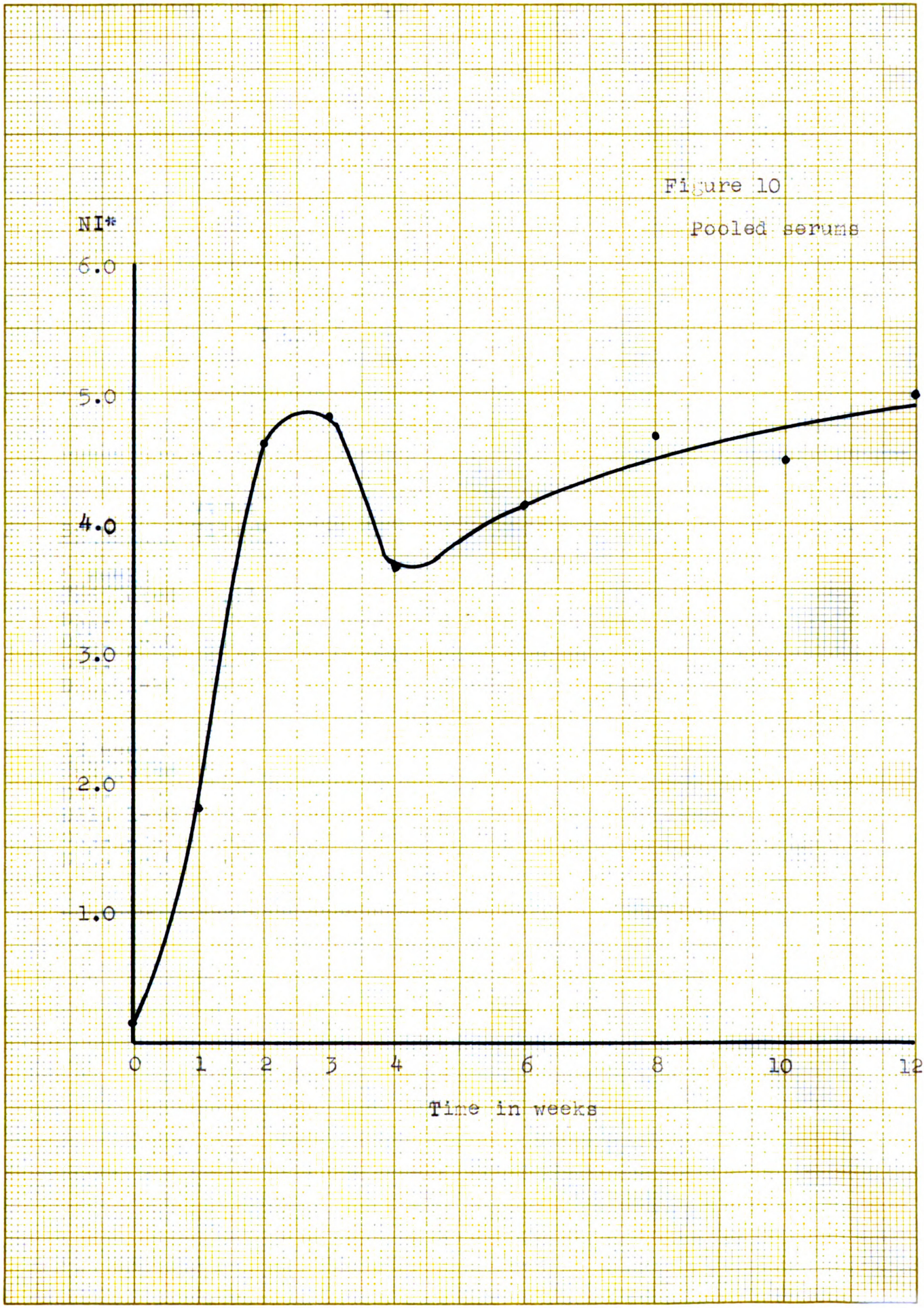


Table 11 -- Effect of dilution on neutralization indices

Serum	Virus Titer*	Virus dilutions 10^{-0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Serum Titer*	NI+
6 weeks									
Undiluted	5.63	5	2≠	1	0			1.22	4.41
Dil. 1-5	5.63		5	4	0	0		2.30	3.25
Dil. 1-10	5.63		5	5	1	0		2.63	3.00
Dil. 1-15	5.63		4≠	5	0	0	0	2.50	3.13
Dil. 1-20	5.63			5	1	0	0	2.63	3.00
12 weeks									
Undiluted	5.63	4	0	0	0			0.38	5.25
Dil. 1-5	5.63	5	3	1	0	0		1.32	4.31
Dil. 1-10	5.63	5	3	0	0	0		1.17	4.46
Dil. 1-15	5.63		4	1	1	0	0	1.62	4.01
Dil. 1-20	5.63			1≠	0	0	0	≧1.66	≦3.97

Results and Discussion

Chickens K1402F2, K1463T3, K1460Z3, K1318B2 and K1507V3 survived to the end of the experiment, but K1462Y3 died from peritonitis, K1457B4 died from a ruptured artery, and K1516F2 died from unknown causes 2, 4 and 9 weeks, respectively, after the beginning of the experiment. As a result, only 3 serum samples were collected from K1462Y3, 4 samples from K1457B4, and 7 samples from K1516F2.

The neutralizing indices of the pre-exposure serum samples and the serums from control chickens, Tables 1 to 8, closely agree with the work of Cunningham[15] who reported $\bar{X}\ 10^{0.389} \pm 10^{0.0376}$, $\sigma\ 10^{0.376}$, or $10^{1.517} \pm 10^{0.0375}$ ($\bar{X} \pm 3\sigma$) as the infectious bronchitis neutralization index for normal chicken serum with V114D antigen. According to these data, 99.7 per cent of the serums from normal chickens should contain not more than $10^{1.5546}$ of 35 neutralizing doses as detected by the serum neutralization test.

One week after exposure to the virus there was a slight increase of the neutralization indices of all serums. From the 2nd to the 6th weeks there was a marked increase of the neutralization indices. The maximum neutralization index occurred at the 6th week followed by a decline at the 8th week, an increase at the 10th week, and a decline at the termination of the experiment at the 12th week. Tables 3 to 8, Figures 3 to 8.

The neutralization indices of the serums showed close

agreement at all intervals. During the initial period of ascending indices a logarithmic pattern was followed. Experimental evidence is not available to explain the subsequent variations in the indices at the 8th, 10th and 12th weeks. Tables 3,4,7,8 and Figures 3,4,7,8. There are several possible explanations for these variances. The declines at the 8th and 12th weeks could possibly be explained on the basis of the interference phenomenon reported by Groupe[23] since this decline was associated with a virus of low titer which occurred when a 24-hour harvest from dead embryos inoculated with V114D was used as the antigen in the neutralization tests. Tables 1,2,7,8 and Figures 1,2,7,8. However, such a possibility can be partially discounted on the basis of the data in Tables 3 and 4. A sample of the virus which had a high titer was used for titrations of the 6th, 8th, and 10th week serums from K14602Z3. The decline and rise of the neutralization indices were observed, but the decline was not as pronounced as that observed for K13102, Table 4 and Figure 4, where the 6th, 10th and 12th week serum samples were titrated with another sample of virus of low titer. Therefore, the decline in neutralization indices could possibly be due to the presence of interfering materials to the extent of the difference between the 8th week neutralization index of K14602Z3 and the 8th week neutralization index of K13102. Figures 3 and 4.

Another possible explanation could be based on sub-

clinical infections. The experimental chickens were maintained in a semi-isolation room and there was the possibility that the chickens might have had a subsequent exposure at the 6th week to infectious bronchitis virus from another source. No symptoms of infectious bronchitis were observed during this period.

There is also the possibility that fluctuations of the neutralization indices are a natural occurrence in this disease. Further investigations of the antibody-antigen reaction should be undertaken.

Jungherr and Terrell[30] reported that passive immunity was transferred via the yolk in eggs laid from immune parent stock. This probably was a passive transfer of neutralizing antibodies since Hofstad and Kenzy[29] were not able to demonstrate immunity but were able to produce the disease in chicks hatched from eggs laid by immune parent stock. The neutralizing titers at the 3rd and 4th weeks as reported by Jungherr and Terrell may be considered as being within the neutralization index range of normal chicken serum reported by Cunningham[15] and on the findings in this experiment. An antibody titer of 1,000 neutralizing doses as reported by Jungherr and Terrell may be sufficient to initially inactivate the virus, but due to the possible reversibility of the serum-neutralization of the virus, and the fact that the virus is in a multiplication phase, this antibody titer might soon be ineffective and infection could result.

Burnet *et al*[7,8] stated that inactivation of certain viruses with immune serum resulted primarily from the union of antibody to the virus surface. This was a reversible union and the time required to reach an equilibrium was approximately proportional to the concentration of the antibody. A permanent antibody-virus union was possible after prolonged incubation.

The Effect of Pooling Immune Serums

Figure 9 shows the average neutralization indices of the serums at the several periods following exposure to infectious bronchitis virus as calculated from the data in Table 9.

It was thought that by pooling aliquots of the serums, results would be obtained that would closely agree with the averages as calculated above. However, the results obtained indicated a considerable deviation and can not be explained at the present time. Further studies of this phenomenon should be undertaken. Table 10, Figure 10.

The Effect of Dilution of Serum on Neutralization

Hirst[24] demonstrated that a dilution of 1 in 5 of influenza immune serum resulted in a 10-fold decrease in neutralizing capacity when tested in mice.

Brandly et al[6] showed that dilution of 1 in 10 of Newcastle disease immune serum resulted in a 100-fold decrease in neutralization capacity, but a dilution of 1 in 5 resulted in a 10-to 100-fold decrease in neutralizing capacity.

Rached[34] found that Newcastle disease immune serum diluted 1 in 10 in nutrient broth resulted in a 20-to 100-fold decrease in the neutralizing capacity.

Table 11 shows the results of serum neutralization tests using serum diluted 1 in 5, 1 in 10, 1 in 15, and 1 in 20 in 0.85 per cent NaCl. Dilution of the immune serum 1 in 5 resulted in a decrease in neutralizing capacity 10-to 15-fold, and further dilutions of the serum did not appreciably alter the neutralizing capacity of the immune serum.

Summary

(a) Normal chickens were exposed to an active field strain of infectious bronchitis virus. The serological response of the chickens at certain time intervals were evaluated by serum neutralization tests with the results expressed as the neutralization index.

(b) Pre-exposure neutralization indices were found to be $10^{1.53}$ or less.

(c) There was found to be a one week period in which no significant production of neutralizing antibodies could be demonstrated by serological tests.

(d) The logarithmic phase of antibody production was found to begin two weeks following exposure and the maximum neutralization index was reached at six weeks following exposure.

(e) Dilution of immune serum 1 in 5 in 0.85 per cent NaCl resulted in a 10-to 15-fold decrease in the neutralizing capacity. Further dilution up to 1 in 20 did not appreciably decrease the neutralizing capacity of the immune serum.

(f) The results of serum neutralization tests on pooled serums indicate the need of further study of antibody-antigen reactions.

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