IN VIVO ALTERATION OF AGGLUTINABILITY OF CHICKEN ERYTHROCYTES BY NEWCASTLE DISEASE VIRUS AND POSSIBLE APPLICATION AS A DIAGNOSTIC AID IN NEWCASTLE DISEASE

by

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INTRODUCTION

A great deal has been learned about viral hemagglutination since the report of Hirst (7) that influenza viruses agglutinate chicken red blood cells. Of special interest have been those experiments that suggested that this reaction may serve as a basisfor the study of viral infections.

This study was planned to determine whether normal chicken red blood cells which have come in contact with Newcastle disease virus *in vivo* are changed in such a way that they are no longer sensitive and will not agglutinate in the presence of fresh homologous virus. Some *in vitro* experiments (3, 4, 5, 6, 14) indicate that there is an alteration in the normal chicken red blood cell when it comes in contact with Newcastle disease virus. These *in vitro* experiments were accomplished by mixing various strains of Newcastle disease virus with normal erythrocytes in the test tube. The virus agglutinated the cells. After washing these cells with a physiological salt solution it was found that they were no longer sensitive or would not agglutinate when fresh virus was added.

This investigation was concerned with an *in vivo* alteration of the agglutinability of the erythrocyte when it comes in contact with Newcastle disease virus. Test groups of chickens were inoculated with Newcastle disease virus and agglutination tests were run, observing the changes in sensitivity or agglutinability of the erythrocytes as the disease progressed.

The ultimate aim of such a study was to apply this change of agglutinability of the chicken red blood cell to a rapid diagnostic test for the presence of Newcastle disease virus.

LITERATURE REVIEW

Hirst (9) reported that erythrocytes that had been agglutinated by influenza viruses, namely PR8 and Lee, and subsequently fully dissociated from them, were no longer capable of adsorbing a detectable amount of fresh virus of either strain or a mixture of the strains or of agglutinating in their presence. He suggested that this hemagglutination phenomenon of the erythrocytes might be an *in vitro* counterpart to natural infection.

Hirst (9 and 11) suggests that the agglutination phenomenon is due to receptors on the red cell. He states that the virus attaches itself to the red cell receptor, and after 4 to 6 hours it is eluted from the cell, removing the receptor by enzymatic action. Therefore there are no receptors left on the cell for the virus, and when fresh virus is added to the resuspended cells there is no agglutination. Smadel (17) states that the agglutination of erythrocytes by viral agents, Hirst phenomenon, has no relation to the classical antigen-antibody reaction.

Following the original description of the phenomenon and its use in the diagnosis of influenza, other workers have demonstrated its applicability to *in vitro* studies of a number of infections. Lush (14) demonstrated that the Hirst red cell agglutination test is applicable to the virus of Newcastle disease and of fowl plague. She further showed by inhibition tests that there is no serological relationship between the viruses. Burnet (3) states there is no serological relation between Newcastle disease virus and influenza viruses. Burnet (4) observed that when test tubes containing red cells agglutinated by Newcastle disease virus were reshaken after standing an hour or two at room temperature, they usually failed to reagglutinate. Cells agglutinated by any of the influenza viruses similarly treated, reagglutinated in the same fashion as

at first. To examine this difference more closely, red cells were agglutinated by an excess of Newcastle disease virus and then washed twice at room temperature with normal saline. When resuspended in saline the washed cells showed the normal stability of untreated cells. This cell emulsion was treated with the various viruses capable of agglutinating normal fowl cells. Influenza A, influenza B, and vaccinia viruses agglutinated the cells in normal fashion; Newcastle disease virus produced no agglutination whatever, although the virus used produced typical agglutination of normal cells.

Florman (6) recently investigated the alteration of chicken erythrocytes treated with influenza and Newcastle disease virus. He states that the loss in adsorptive capacity is proportional to the loss in agglutinability of red blood cells, when treated with either homologous or heterologous virus. The modifications in erythrocytes that follow treatment with Newcastle disease virus are not correlated with changes that follow infection of chick embryos by this virus. This phenomenon is demonstrated by the fact that red blood cells which have been treated with Newcastle disease virus are still capable of adsorbing and being agglutinated by the PR8 strain of influenza. However, chick embryos which have been infected by Newcastle disease virus 24 hours previously are no longer susceptible to infection with a lethal dose of PR8 strain of influenza virus.

Hofstad (13), attempting to utilize the chicken red cell agglutination test as a diagnostic aid for infectious bronchitis, found that infectious bronchitis virus did not agglutinate normal chicken blood cells.

A recent <u>in vivo</u> investigation demonstrates the role that the

hemagglutination test may play in the diagnosis of viral infections. Moses, Brandly, Jones, and Jungherr (15) state that the test may be of value in the diagnosis of fowl plague and Newcastle disease. They found that red cells infected by the plague virus were agglutinated neither by materials containing the homologous virus nor by the heterologous viruses. In their study they used the Dutch East Indies strain of plague virus, the Hertfordshire, and 3 California strains of Newcastle disease virus, and the PR8 strain of influenza virus. They demonstrated also that red cells infected with the Newcastle virus strains were insensitive to the materials containing the same or different strain of that virus, but were fully sensitive to the plague virus and somewhat sensitive to the influenza virus. This investigation demonstrates that erythrocytes infected with Newcastle disease virus or fowl plague are insensitive to the homologous virus, and the possibility of using the hemagglutination test as a diagnostic aid in Newcastle disease.

The fact that Newcastle disease virus may be found in the blood was brought out by the study of Brandly, Moses, Jungherr, and Jones (1) who investigated the presence and concentration of Newcastle disease virus in the feces, cleft palate, mouth secretions, and blood. They found the highest demonstrable virus concentration was present in the feces and blood cells. The virus was present more consistently and in greater quantity in the blood cells than in the plasma. Another important factor found was that the virus content of the serum and blood was greater with some strains of Newcastle disease than with others.

Brandly, Hanson, Lewis, Winslow, Hoyt, Pritchard, and Merlinger (2) made an investigation of the agglutinability of normal chicken red blood cells, using White Leghorns, New Hampshire Reds, and Barred Rocks in their

test group. The results indicate that it is normal to expect a considerable variation in agglutinability among chicken red blood cells of different individuals. They further point out that red cells from successive bleedings of a single bird, over a period of weeks, tend to give a rather constant titer with a given lot of virus. Red blood cells from seven of the ten birds tested had a variation of only one tube dilution in three successive titrations made over a period of a month.

In another experiment these same investigators (2) state that virus can be isolated from the erythrocytes through the seventh day after infection. In their investigation they used an attenuated Newcastle virus (New Jersey K and D) strain, inoculated intratracheally. Florman (6) in his studies on alterations in chicken erythrocytes with Newcastle disease virus states that the adsorptive capacity and agglutinability modifications in such treated red blood cells may persist for as long as 21 days.

METHODS

From a careful review of the literature, it appears that only one study has been made on *in vivo* alteration of chicken erythrocytes, in which blocking of the hemagglutinin action occurred in red cells from embryos infected with both homologous and heterologous viruses (15). Some preliminary work at the Virginia Agricultural Experiment Station (12) indicates there is an alteration in red cell agglutination after chicks have been exposed to Newcastle disease.

This investigation was divided into two parts for the purpose of comparing *in vivo* alteration of the agglutinability of chicken red cells. The first part was concerned with tube agglutination of the red cells by the viral agglutinating agent. The latter part dealt with the agglutination of citrated or whole blood by the viral agglutinating agent on a glass plate.

A. Tube agglutination

1. Materials and apparatus

Virus Three strains of Newcastle disease virus (NDV) which differed in their pathogenicity for chicks were selected for study. One was a strain that manifested primarily respiratory symptoms, obtained from Dr. F. R. Beaudette at the New Jersey Experiment Station, and known as the New Jersey KD strain. It had been given the strain designation of N1 at the Virginia Agricultural Experiment Station and as such was used by the author. This strain had had its 14th egg passage. The second strain referred to in the investigation as N2, gave marked nervous symptoms. It was isolated by Dr. J. R. Beach of California and is known as California strain 11914. This had been through six egg passages. The last strain, B1, a strain which

produces very mild symptoms, was obtained from Dr. F. R. Beaudette of the New Jersey Experiment Station. This had had its 75th egg passage.

Preparation of virus strains The method used was a modification of Hirst's technique (10). All of the virus strains were prepared by inoculating 0.1 ml. of infected allantoic fluid into the allantoic sacs of 11 day old chick embryos. After 48 hours incubation at 37°C the eggs were placed at 4°C overnight, and blood free allantoic fluid was removed the following day. If necessary the cellular debris was removed by a short centrifugation, and the viruses were stored at 4°C in the fluid state. The same lot of virus was used throughout for each test. None of the virus used was more than a month old. These strains of viruses were formolized with 0.1% formalin a week before use. Viruses of Newcastle disease may be inactivated by formalin and yet retain hemagglutinating power (5).

Red Cells Chicken blood was collected in sterile test tubes. Approximately 1 ml. of blood was obtained from the wing vein of chickens. This was drawn into a syringe containing 0.4 ml. of a 2% solution of sodium citrate. The cells were separated from the citrated plasma by centrifugation and washed three times with quantities of physiological salt solution equal to 5 to 10 times the volume of packed cells. The washed cells were then resuspended in fresh salt solution, transferred to a graduated tube and centrifuged for exactly 10 minutes at 1500 r.p.m. The volume of packed cells was readily estimated and a 10% suspension was made by adding sufficient salt solution to reach a volume 10 times that of the packed cells. These were stored at 4°C. According to Salk (16) such a suspension will contain from 16,000 to 17,000 cells per mm^3. This has been determined with the Sahli hemoglobinometer.(16). Just before use a 0.5% suspension of

these cells was made. In no case was a suspension kept longer than a week. Almost all the titrations were accomplished with new cells and fresh suspensions since one of the objectives was to note early changes in the cells.

Hemagglutination A modification of Hirst's test (8) was used throughout for the virus-cell titrations. Briefly, this consists of mixing in chemically clean, round bottom Pyrex test tubes 0.25 ml. of 0.85% salt solution, 0.25 ml. of the desired dilution of the virus and 0.25 ml. of the 0.5% cell suspension. These ingredients are thoroughly mixed by vigorous shaking. The mixture is allowed to stand at room temperature and readings made at 15 minute intervals for one hour, against a good source of light.

Reading the test Observations were made at 15, 30, 45, and 60 minutes. The earlier readings were made because hemagglutination may occur in the first tubes rather quickly but disappear before the final readings were made. A reading rack, using a mirror at a 45° angle beneath the tubes with a gooseneck lamp directly over the racks was used for reading the test results. In tubes in which no agglutination occurs, the cells settled as a central, sharply demarcated round disc. Where agglutination was maximal, a pink film covered the entire bottom of the tube as if the clumps adhered to the bottom of the tube at the point of settling. Intermediate reactions usually appear as an irregular clump with a halo of finely aggregated or unagglutinated cells.

2. Procedure

Some preliminary titrations were made on cells collected from normal chickens, using B1, M1, and M2 strains of virus in the test. One of the

over-all objectives of this investigation was to ascertain which of the virus strains used was the best agglutinator. The highest titer was obtained when B1 strain of the virus was used, but good agglutination was also obtained with the N1 strain.

After these preliminary titrations it was decided to inoculate groups of young chickens with the various strains of virus under investigation. Twenty young chickens of various breeds were used in the experiment, but the data included in this report concerns only fifteen, because five of the birds succumbed to the disease before the test in hand was completed. Various groups of chickens were inoculated intranasally with one or two drops of allantoic fluid containing B1, N1, N2 strains of virus. Blood was drawn subsequent to inoculation and every second day thereafter for a period of eight to ten days. The hemagglutination tests were made with a 0.5% suspension of the cells, 24 to 48 hours old that had been stored at 4°C. Cells from each chick were titrated with each of the three strains of virus in doubling dilutions and the same technique of readings was used for all tests. This same procedure was repeated with each lot of cells during the course of the experiment.

B. Plate agglutination

1. Materials and apparatus

Virus The viruses used in this part of the investigation were the same three strains that were used in the tube agglutination method, namely B1, N1, and N2.

Preparation of virus All of the virus strains were prepared as in part A except for the fact that they were formalized with various concentrations of formalin. Lots of each of the virus strains were formalized a week

before use. The same lot of virus was used throughout for each test.

Red cells In part of the rapid-plate agglutination test blood was drawn from the wing vein of chickens into a 2% solution of sodium citrate. In another part of the experiment, in which ten chickens were used, a puncture was made in the wing vein and whole blood was used.

Agglutination A drop of blood, approximately 0.004 ml., either citrated or whole was mixed with 0.03 ml. of the various virus strains on a plate glass, over a light source. This plate was divided into squares of 2½ cm. The loop used to remove the citrated or whole blood was made of nichrome wire gauge 26 (Brown and Sharp), with a diameter of 0.4 cm. The drop of blood was mixed with the loop twenty-five times. The plate was then gently rotated and after a few seconds the test was read.

Reading the test Arbitrary units of -, +, 2+, 3+, and 4+ were chosen for no agglutination to maximal agglutination. The arbitrary units of 4+ through 2+ were based on the gradation of the size of the cell clumps on agglutination. Very fine agglutination, just discernible with the naked eye, was called plus. No agglutination whatsoever was called negative. The temperature of the agglutinating plate was kept at approximately room temperature and care was taken not to get the plate too hot from the light source. Heat appeared to inhibit agglutination, because it tended to dry up the virus and blood before they could agglutinate.

2. Procedure

In employing the plate method it was decided to use either citrated or whole blood from twenty-six chicks four to eight weeks of age. Blood was drawn from the wing vein, either by syringe or needle puncture and one

drop of this was mixed on a glass plate with 0.05 ml. of the virus strain under investigation.

Experiment 1. N1 virus strain Four chicks from individual hens were chosen. The numbers PC42C, PC45B, PC47B, and PC48A represent chicks, eight weeks of age, from hens numbered 42, 45, 47, and 48, respectively, as shown in Tables IV and V. These birds were bled for four successive days to see if there was any variation in the agglutinability of their blood. Citrated blood was used. The chicks were then inoculated intranasally with N1 strain of NDV and bled for seven successive days. Shortly after they were removed to a Newcastle isolation building where they were bled twice a week. These birds were bled nineteen times during a period of thirty-seven days.

For the first two weeks of bleeding all virus strains used as the viral agglutinating agent, namely B1, N1, and N2, were treated with 0.05%, 0.10%, and 0.15% formalin. This was done in order to ascertain the agglutinability of the three virus strains under investigation (see Tables IV and V).

Experiment 2. Replicate of N1 virus strain In order to try and duplicate the above experiment four chicks, four weeks of age, wing band numbers PC43C, PC46A, PC47C, and PC47D, were chosen. They were bled two days before inoculating intranasally with N1 strain of NDV, then daily for a period of a week when they were removed to the Newcastle isolation house, where they were bled twice a week. Citrated blood was used. Virus strains B1 and N1 treated with 0.10% and 0.15% formalin were used as the viral agglutinating agent (see Table VI).

Experiment 3. Replicate of N1 virus strain Since the ultimate aim of

this investigation was the possible applicability of a plate method as a diagnostic aid for Newcastle disease, whole blood from a wing vein puncture was used in place of citrated blood. Five chicks, four weeks of age, wing band numbers 2PC43E, 2PC43F, 2PC45K, 2PC45M, and 2PC49F were chosen. They were bled two days before inoculating with N1 strain of NDV, then daily for a period of ten days, except for the first day after inoculation. Virus strains B1 and N1 formalized with 0.10% and 0.15% formalin were used as the viral agglutinating agent (see Table VII).

Experiment 4. B1 virus strain Plate agglutination tests were run on citrated blood taken from four chicks, six weeks of age, wing band numbers PC43B, PC45C, PC45D, and PC47E, which were inoculated with B1 strain of NDV. Blood was drawn two days subsequent to inoculating with the B1 strain. The birds were then bled for five successive days when they were removed to the Newcastle isolation house where they were bled twice a week for two weeks. B1 and N1 strains of NDV treated with 0.10% and 0.15% formalin were used as the viral agglutinating agent.

Experiment 5. Replicate of B1 virus strain This plate agglutination experiment was performed with whole blood taken from five chicks four weeks of age. Numbers 2PC43H, 2PC45L, 2PC46B, 2PC48E, and 2PC49D represent chicks from individual hens. They were bled two days before inoculating with B1 strain of NDV, except for the first day after inoculation. Virus strains B1 and N1 treated with 0.10% and 0.15% formalin were used as the viral agglutinating agents (see Table IX).

Experiment 6. NB virus strain It was decided to inoculate four chicks, four weeks of age, with NB strain of NDV. This was the most virulent

strain used in the investigation. Chicks with wing band numbers PC43A, PC43E, PC47B, and PC48B were chosen. They were bled two days, then inoculated with NE strain of NDV. Citrated blood was used in the plate agglutination tests. The bleedings were continued for a week, at which time all birds had died of the disease.

RESULTS AND DISCUSSION

An investigation of *in vivo* alteration of the agglutinability of chicken erythrocytes infected with three strains of Newcastle disease virus has been made. In order to do this washed erythrocytes were agglutinated by three viral agglutinating agents by the tube agglutination method, and either citrated or whole blood was agglutinated by the viral agents by the plate technique.

A. Tube agglutination

The data indicate (at 0 days in Figures 1-15 and at 0 days in Tables I-III) that variation in titer is found among non-infected chicken red cells from different individuals. After infection, red cells from successive bleedings of a single bird over a period of two to eight days tends to decrease in titer until a minimum was reached. After the cells reached this minimum in titer they tend to return to normal (see Figures 1-15).

As has been mentioned previously, this agglutination phenomenon is not a classical antigen-antibody reaction, but in this investigation it appeared that the infected red blood cells were altered in such a way that they were no longer sensitive to agglutination by Newcastle disease virus as in the normal or non-infected erythrocytes. A decrease in cell titer or agglutinability is shown graphically (Figures 1-15) in all fifteen of the birds tested, but not with each virus strain employed in the test.

Tables I-III give the tube agglutination titers of red blood cells from each of the fifteen chickens, titrated with B1, N1, and N2 virus strains, respectively. It will be noted that in almost all of the chickens the titers of the second, fourth, or sixth days after infection

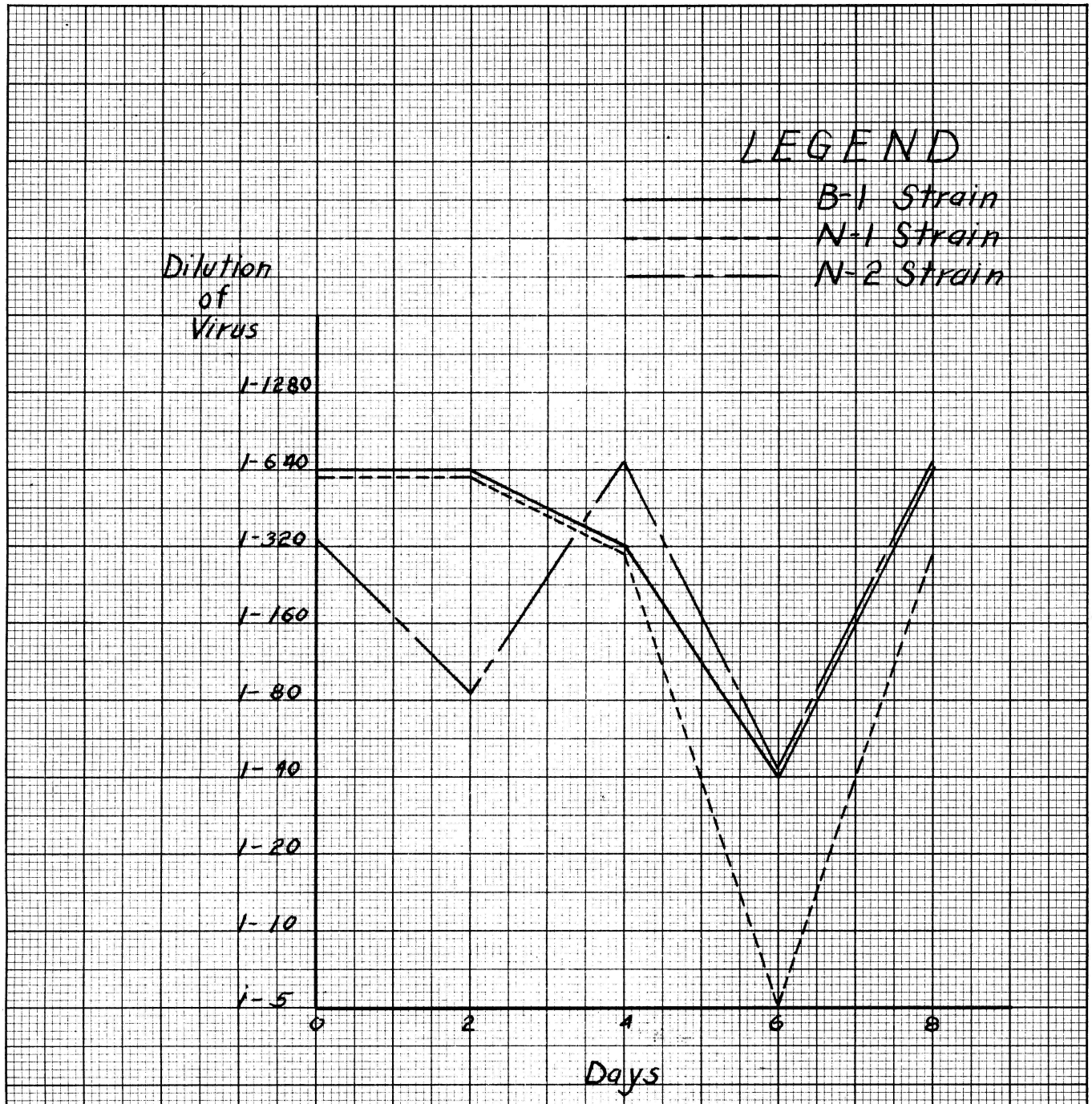

Figure 1 Tube Agglutination Titer of Three NDV Strains With Red Cells Taken From Chick # NPL 44 Inoculated With B-1 Strain.

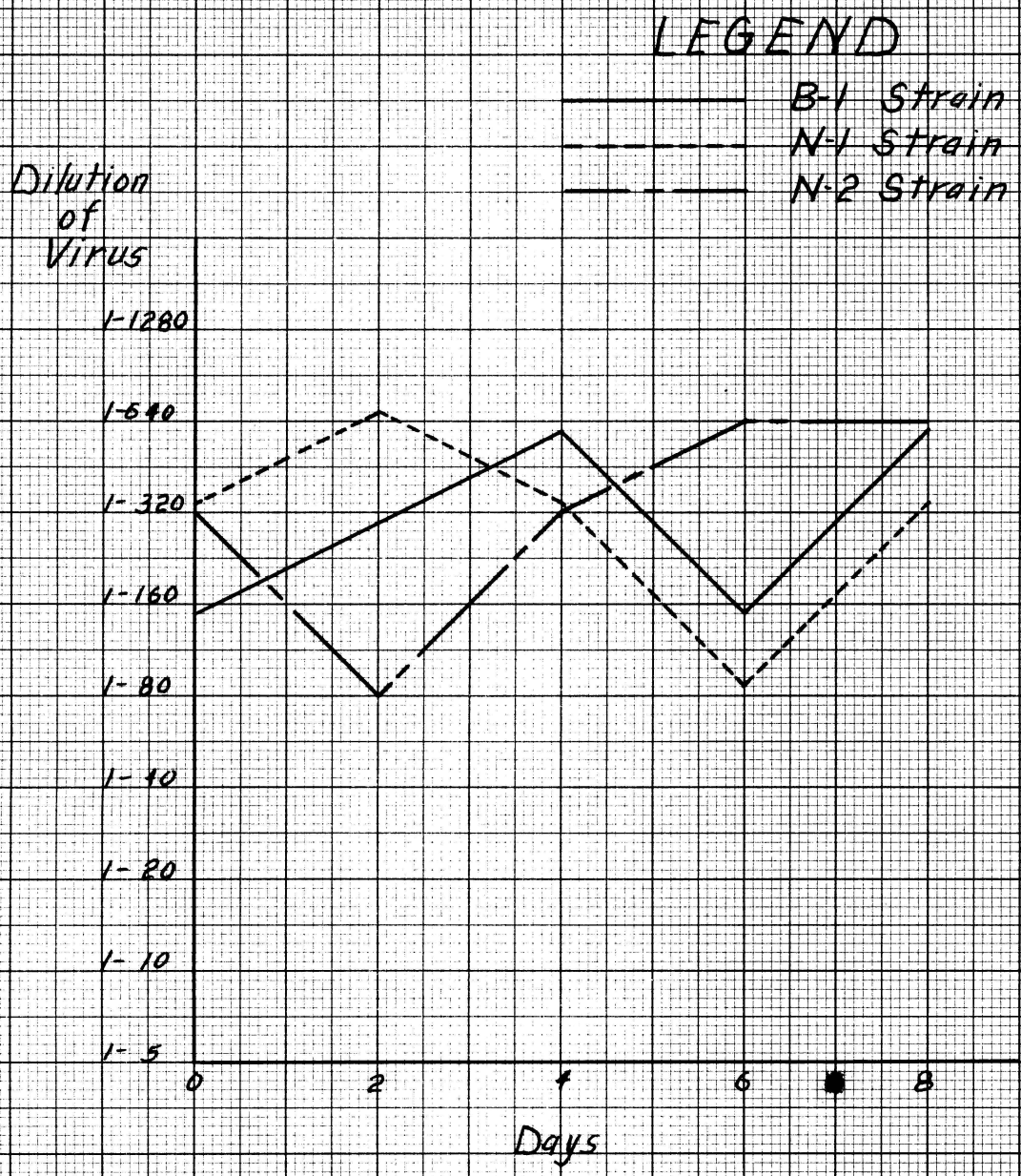

Figure 2 Tube Agglutination Titer of Three NDV Strains With Red Cells Taken From Chick # NPL 46 Inoculated With B-1 Strain.

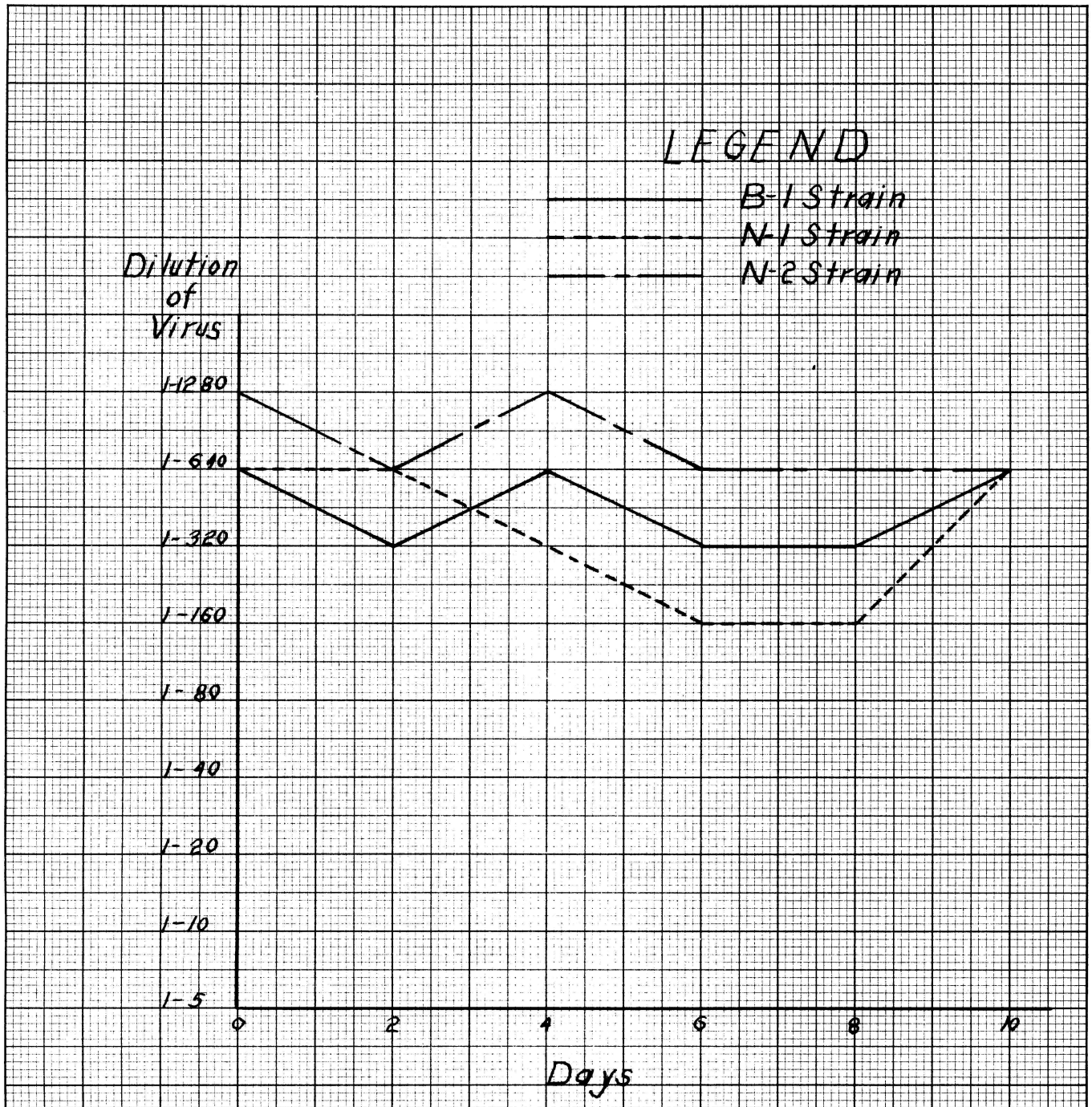

Figure 3 Tube Agglutination Titer of Three NDV Strains With Red Cells Taken From Chick # 94 Inoculated With B-1 Strain.

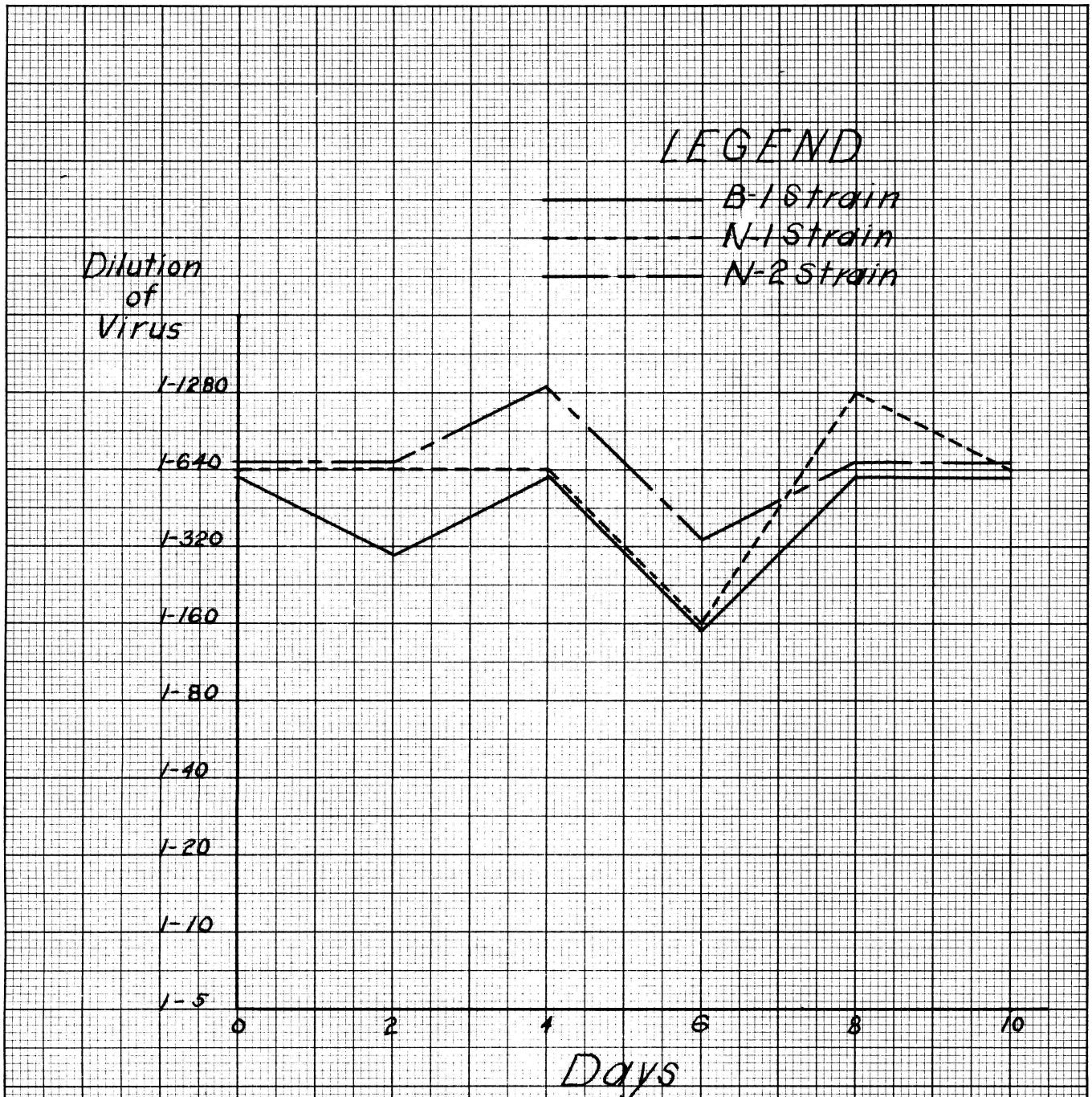

Figure 4 Tube Agglutination Titer of Three NDV Strains With Red Cells Taken From Chick # NPL 123 Inoculated With B-1 Strain.

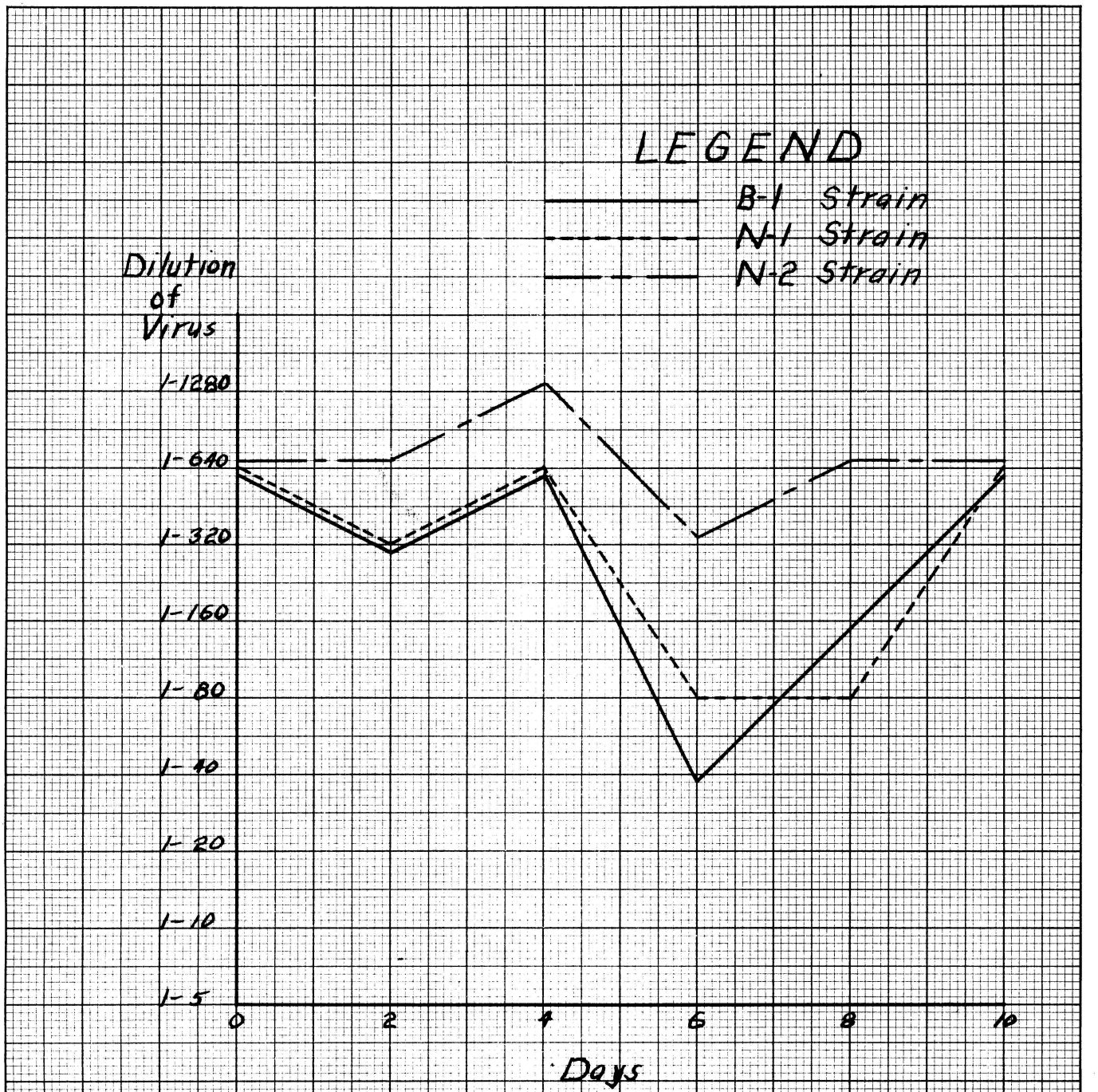

Figure 5 Tube Agglutination Titer of Three NDV Strains With Red Cells Taken From Chick# NPL 126 Inoculated With B-1 Strain.

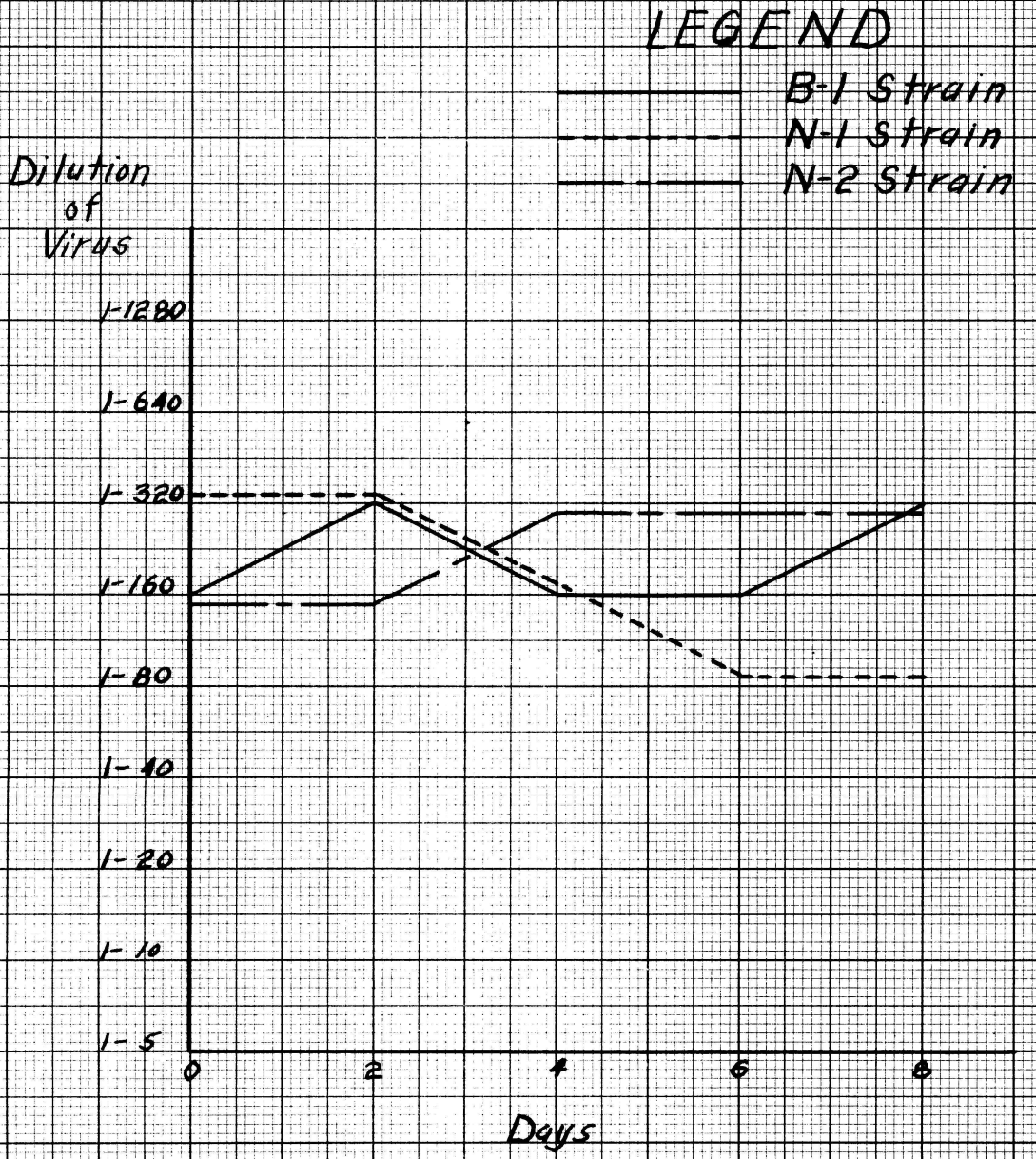

Figure 6 Tube Agglutination Titer of Three NDV Strains With Red Cells Taken From a Rock Chick Inoculated With B-1 Strain.

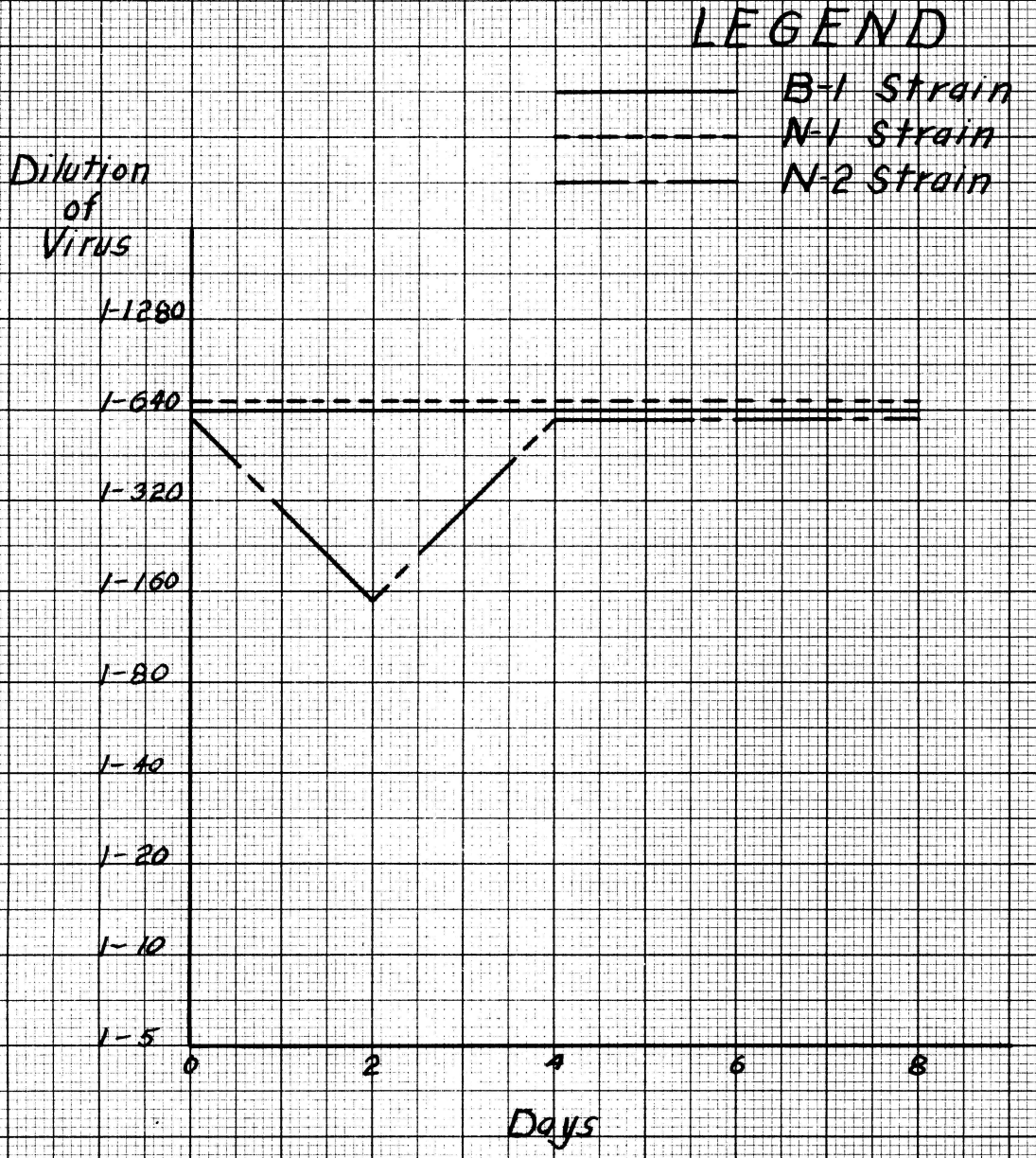

Figure 7 Tube Agglutination Titer of Three NDV Strains With Red Cells Taken From Chick # NPR 57 Inoculated With B-1 Strain.

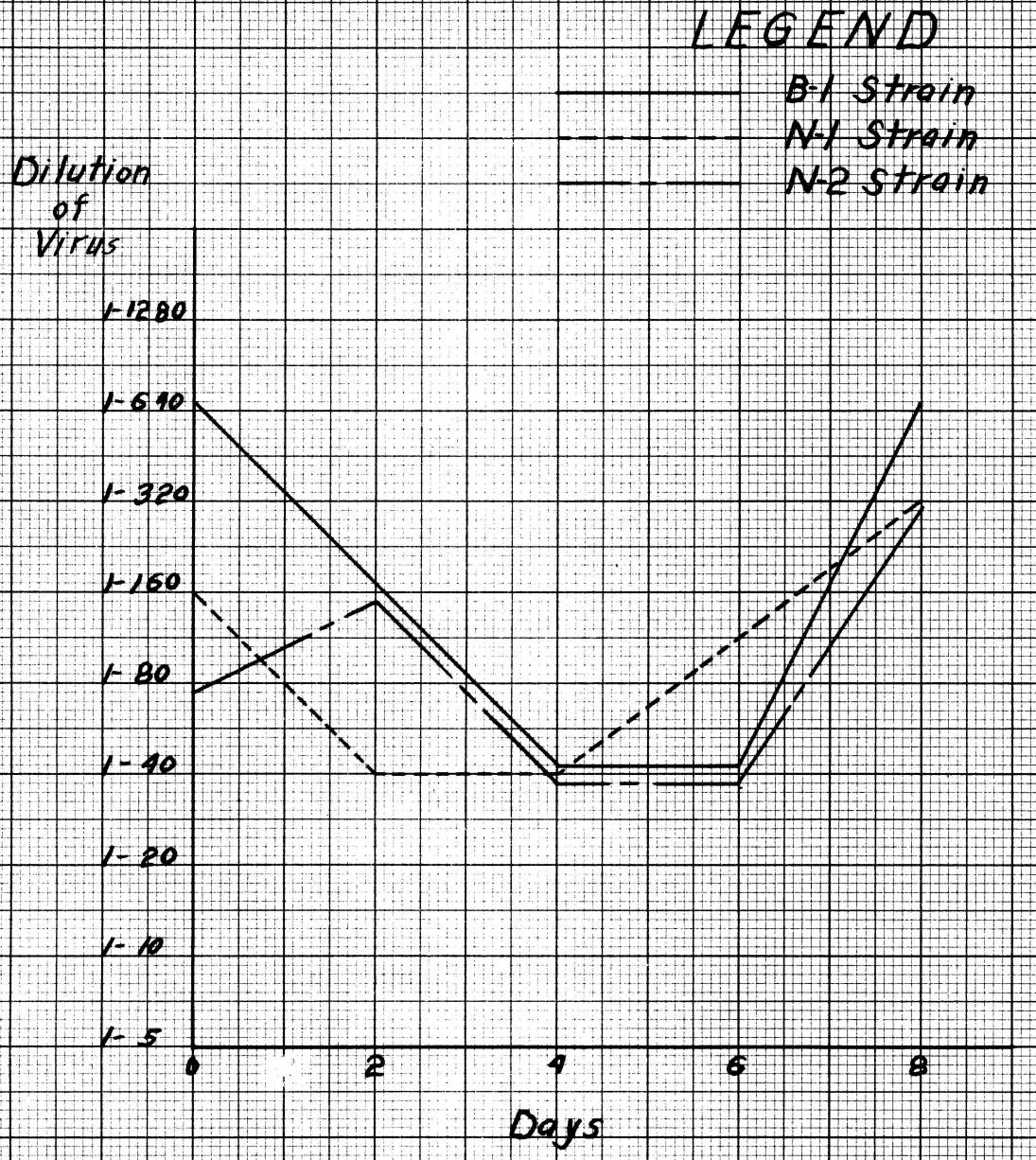

Figure 8 Tube Agglutination Titer of Three NDV Strains With Red Cells Taken From Chick # B 56 G 2 Inoculated With N-1 Strain.

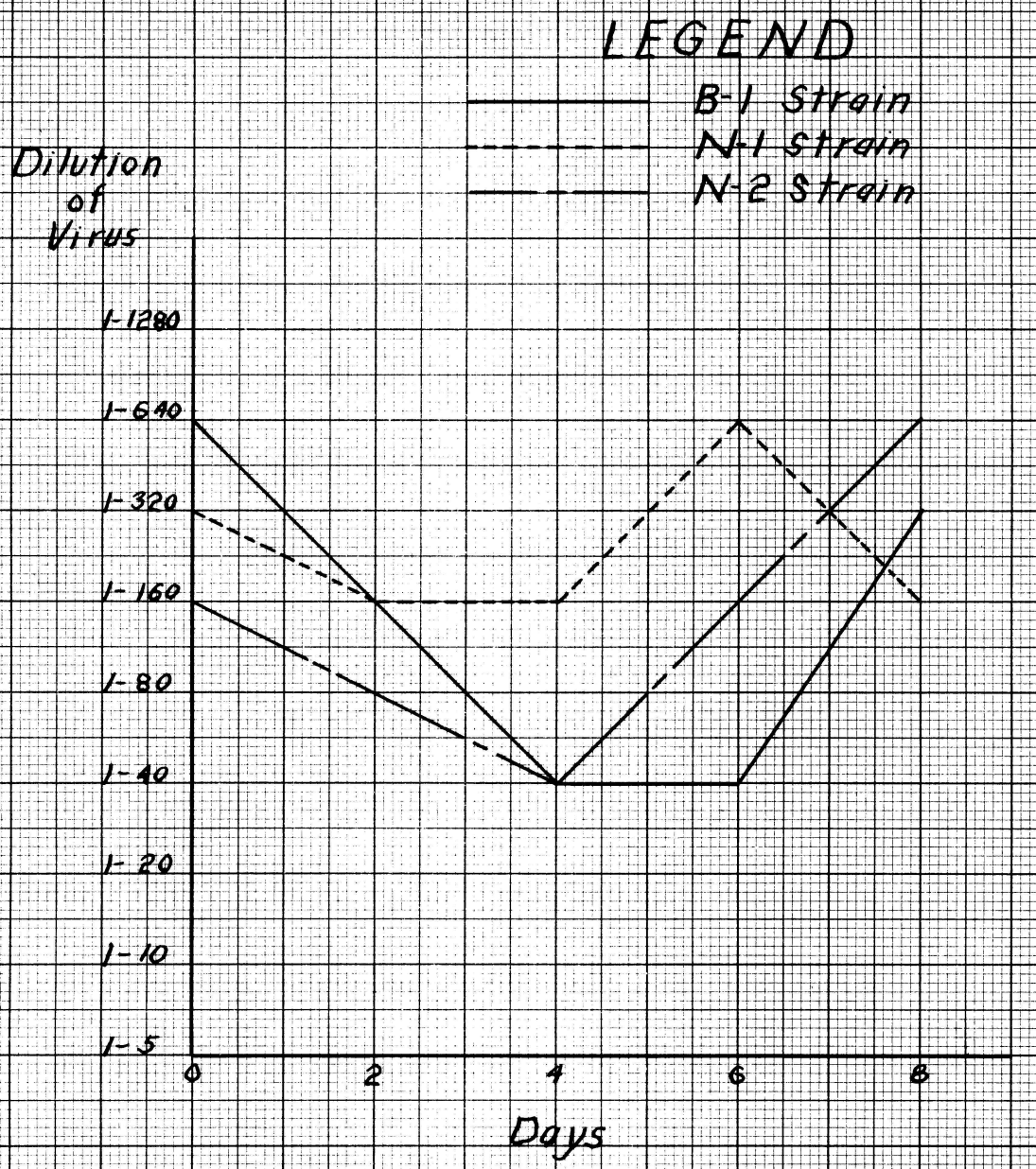

Figure 9 Tube Agglutination Titer of Three NDV Strains With Red Cells Taken From Chick *B-65D-2 Inoculated With N-1 Strain.

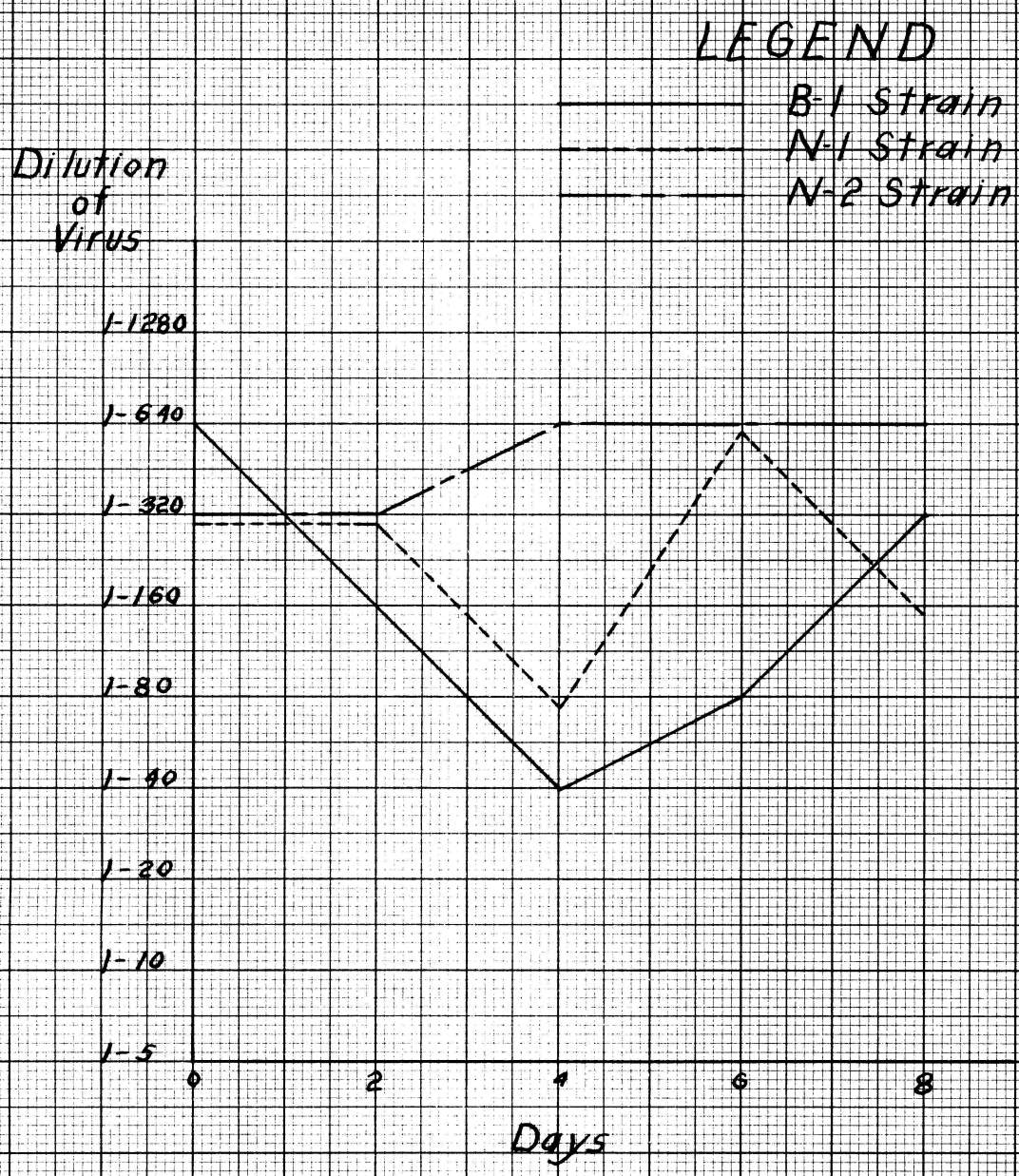

Figure 10 Tube Agglutination Titer of Three NDV Strains With Red Cells Taken From Chick # B67H2 Inoculated With N-1 Strain.

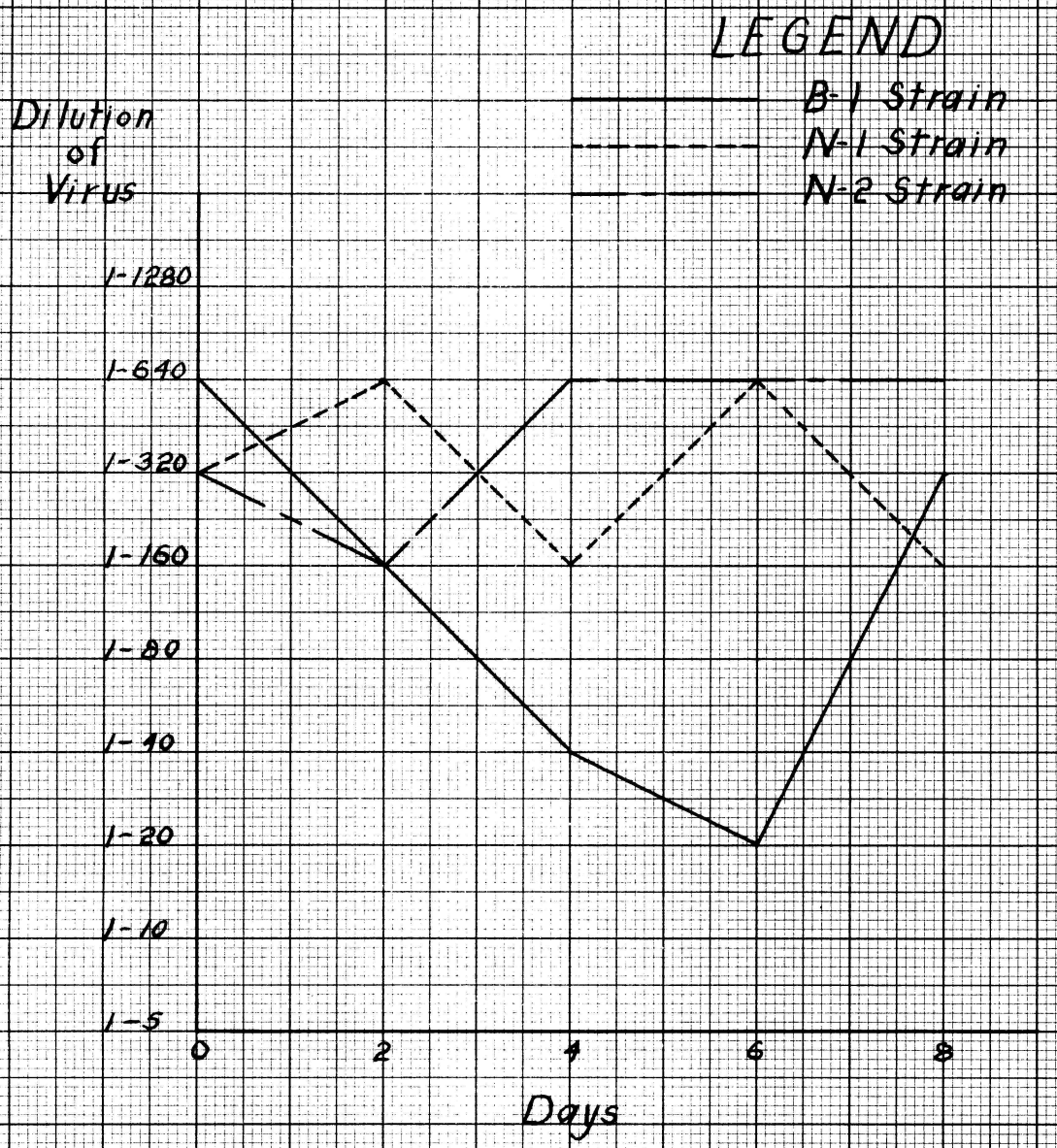

Figure 11 Tube Agglutination Titer of Three NDV Strains With Red Cells Taken From Chick # B 78 H2 Inoculated With N-1 Strain.

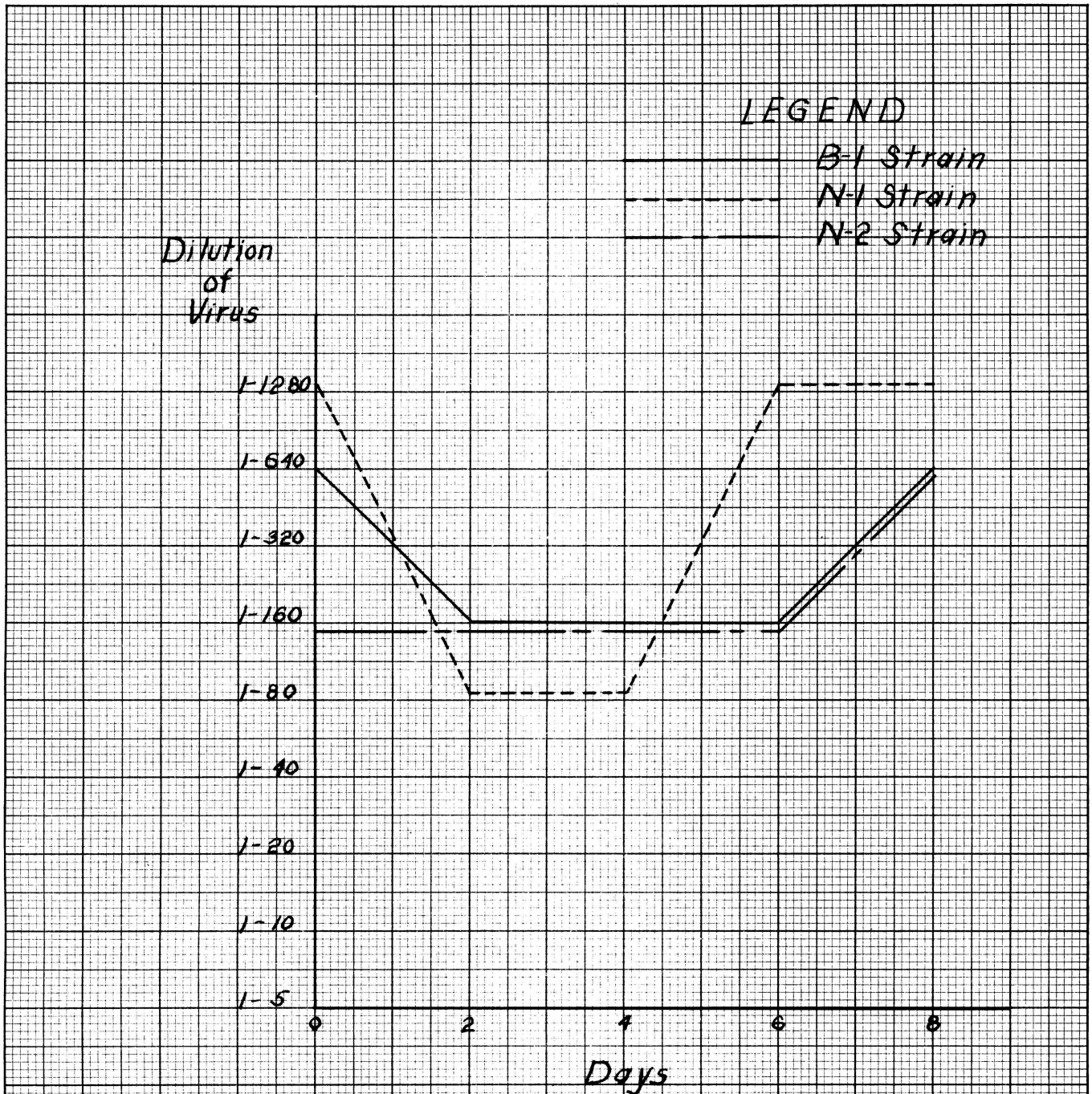

Figure 12 Tube Agglutination Titer of Three NDV Strains With Red Cells Taken From Chick #1808 Inoculated With N-2 Strain.

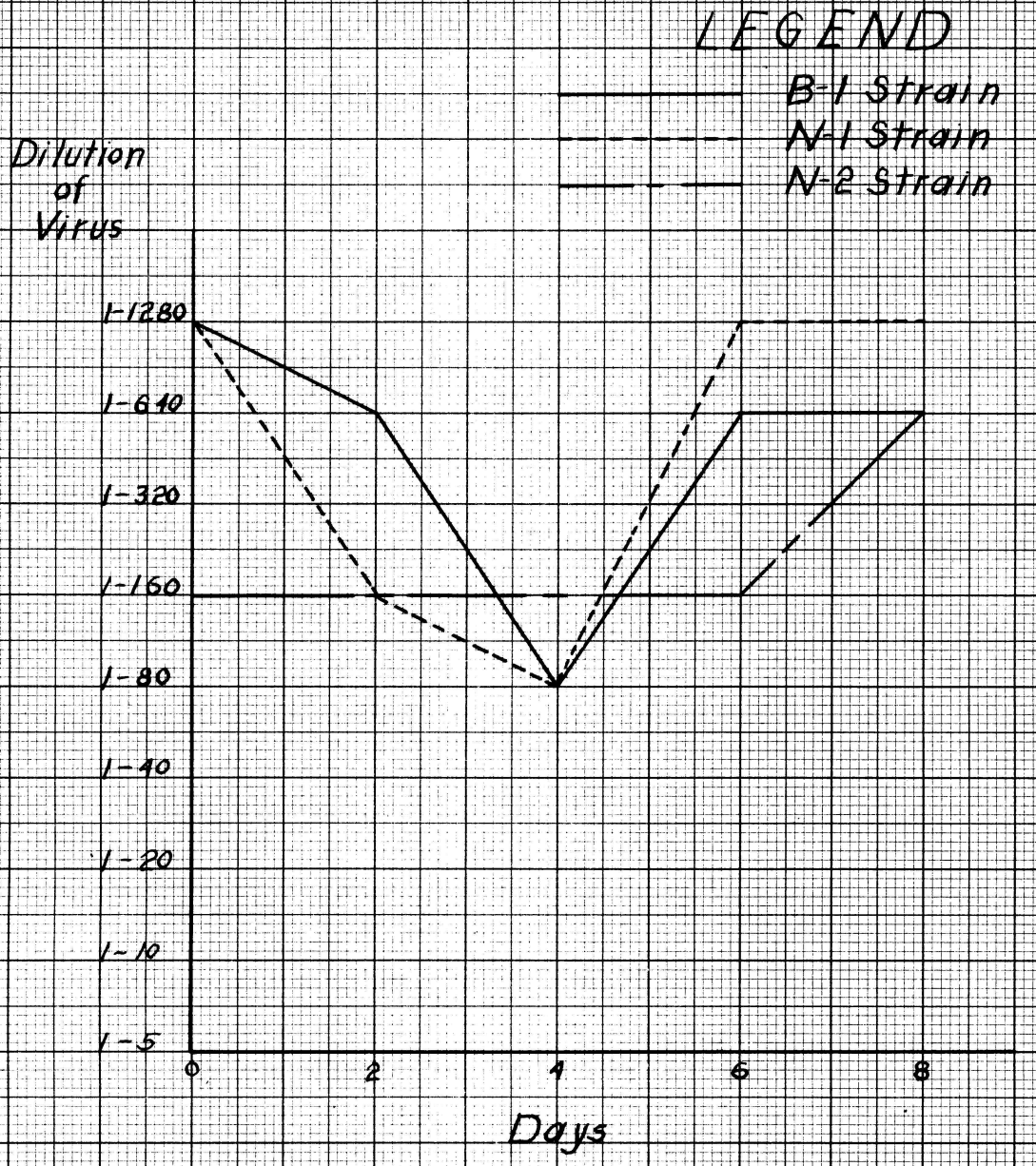

Figure 13 Tube Agglutination Titer of Three NDV Strains With Red Cells Taken From Chick # 1871 Inoculated With N-2 Strain.

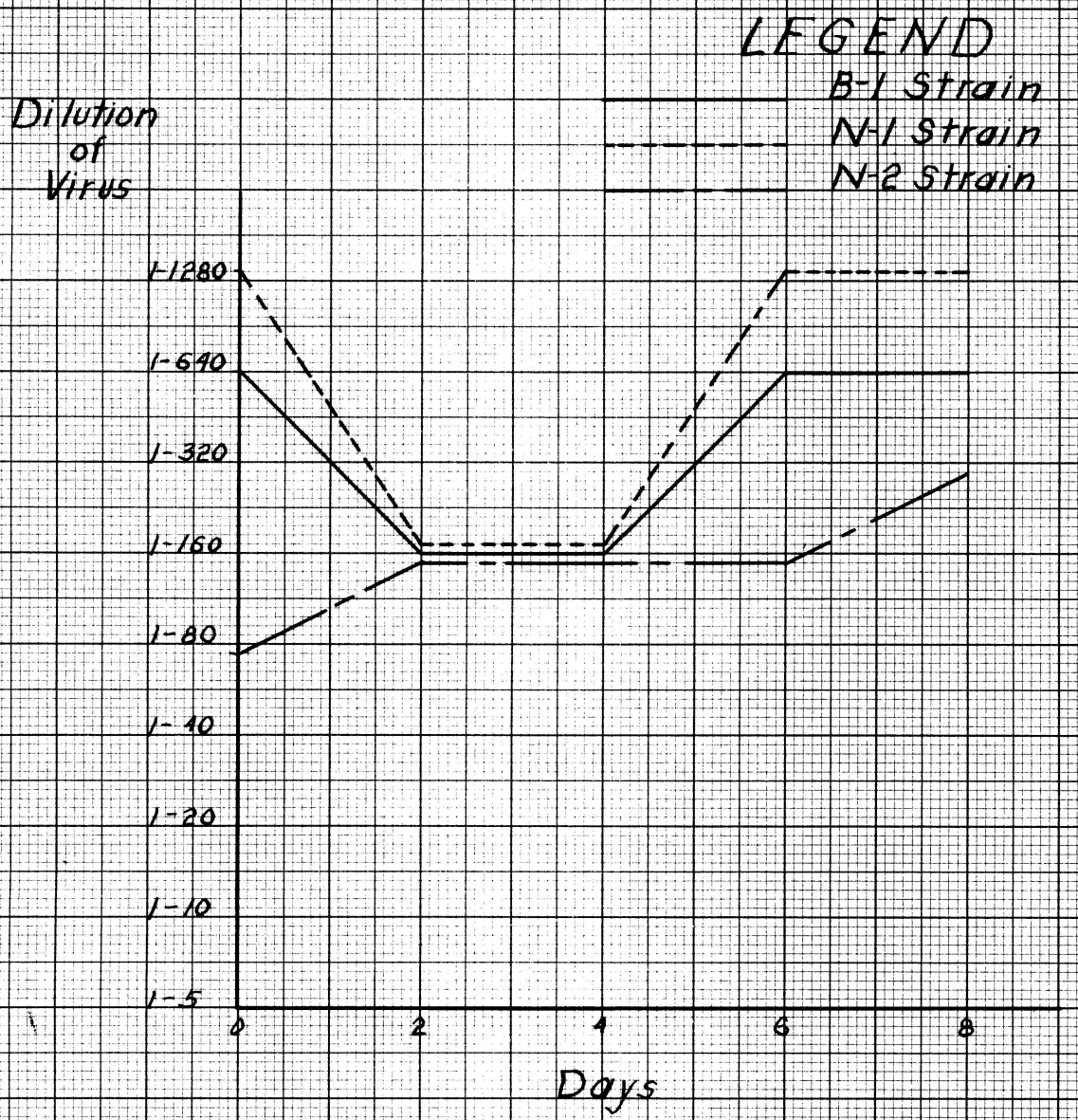

Figure 14 Tube Agglutination Titer of Three NDV Strains With Red Cells Taken From Chick # 1879 Inoculated With N-2 Strain.

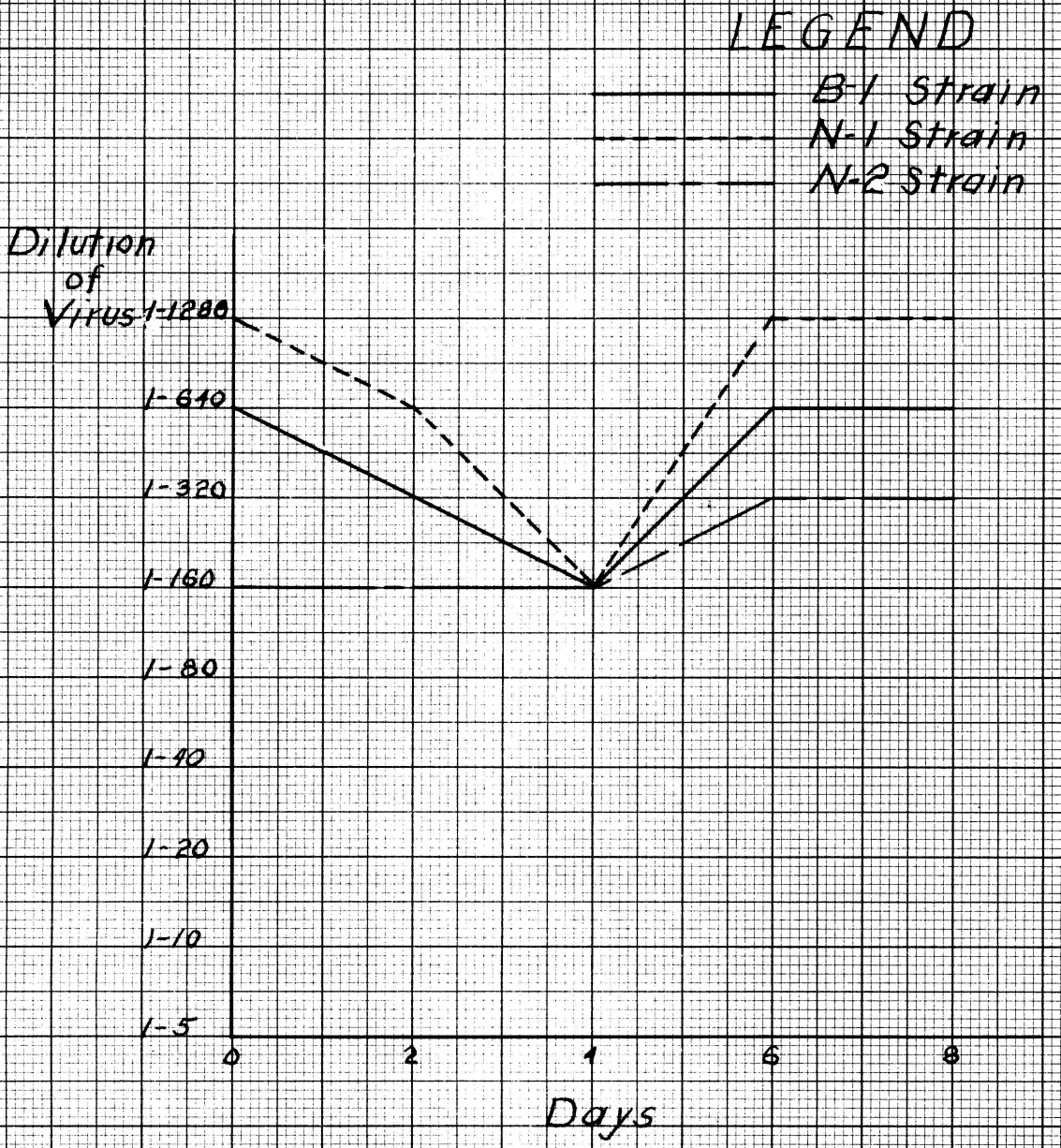

Figure 15 Tube Agglutination Titer of Three NDV Strains With Red Cells Taken From Chick # 1093 Inoculated With N-2 Strain.

Table I.

Tube agglutination titers of chicken red blood cells with B1 strain of Newcastle disease virus

Chicken number	Days after inoculation					
	0	2	4	6	8	10
NPL 94	640	320	640	320	320	640
NPL 123	640	320	640	320	640	640
NPL 126	640	320	640	40	160	640
NPL 44	640	640	320	80	640	
NPL 48	160	320	640	160	640	
1808	640	160	160	160	640	
1871	1280	640	80	640	640	
1879	640	160	160	640	640	
1895	640	320	160	640	640	
D6602	640	160	40	160	640	
D6502	640	160	40	40	320	
D6702	640	160	40	80	320	
D7002	640	160	40	80	320	
NPR 57	640	640	640	640	640	
ROCK	160	320	160	160	320	

Table II.

Tube agglutination titers of chicken red blood cells with M1 strain of Newcastle disease virus

Chicken number	Days after inoculation					
	0	2	4	6	8	10
NPL 96	640	640	320	160	160	640
NPL 123	640	640	640	160	1280	640
NPL 126	640	320	640	80	80	640
NPL 44	640	640	320	5	320	
NPL 48	320	640	320	80	320	
1806	1280	80	80	1280	1280	
1871	1280	160	80	1280	1280	
1870	1280	160	160	1280	1280	
1893	1280	640	160	1280	1280	
B66G2	160	40	40	80	320	
B65H2	320	160	160	640	320	
B67H2	320	320	80	320	160	
B78H2	320	640	160	640	320	
NPH57	640	640	640	640	640	
ROCK	320	320	160	80	80	

Table XII.

Tube agglutination titers of chicken red blood cells with N2 strain of Newcastle disease virus

Chicken number	Days after inoculation					
	0	2	4	6	8	10
NPL 94	1280	640	1280	640	640	640
NPL 125	640	640	1280	320	640	640
NPL 126	640	640	640	80	320	640
NPL 44	320	80	640	40	640	
NPL 48	320	80	320	640	640	
1808	160	160	160	160	640	
1871	160	160	160	160	640	
1879	80	160	160	160	320	
1893	160	160	160	320	320	
B6502	80	160	40	40	320	
B6502	160	80	40	160	640	
B6702	320	320	640	640	640	
B7802	320	160	640	640	640	
NPN57	640	160	640	640	640	
ROCK	160	160	320	320	320	

are lower than at any other time. This indicates again, that as infection of each of the three virus strains progressed the red cells seemed to lose their sensitivity for Newcastle disease virus.

B. Plate agglutination

Experiment 1. M1 virus strain From Tables IV and V it may be seen (at 0 days) that the degree of agglutinability of non-infected erythrocytes was fairly constant from day to day for the viral agglutinating agents, but it was particularly true of the M1 virus strain treated with 0.10% and 0.15% formalin. After twelve bleedings it was demonstrated that the M2 virus strain treated with all concentrations of formalin was unsatisfactory as the viral agglutinating agent to be used in the rapid-plate method. Erythrocytes from all birds were insensitive to it at one time or another during the four day bleeding period before inoculation. Also, the M1 and N1 strains treated with 0.05% formalin were unsatisfactory, as the normal cells from all four birds were insensitive to it on one day or another. Because M2 virus strain treated with all concentrations of formalin and N1 and B1 strains treated with 0.05% formalin were unsatisfactory as viral agglutinating agents they were no longer used in the investigation.

Symptoms of the disease and sensitivity change of the red cells appeared on or about the same day, in three of the four birds used. On the fifth and sixth day after inoculation red cells from two of the birds were insensitive to the viral agglutinating agents. On the tenth day after infection chicken number P045B succumbed to the disease. When the erythrocytes became insensitive or nearly so they remained insensitive for a period of ten days to two weeks, when their sensitivity began

TABLE IV.

Degree of agglutination of erythrocytes, by plate method,

from chicks* inoculated with N1 strain of Newcastle disease virus

Chick Number / Virus Strains / Conc. Formalin in % / Days after inoculation	PC42C									PC45B								
	B1			N1			N2			B1			N1			N2		
	.05	.10	.15	.05	.10	.15	.05	.10	.15	.05	.10	.15	.05	.10	.15	.05	.10	.15
0	3+	3+	3+	2+	3+	3+	-	2+	+	-	4+	4+	-	3+	3+	-	+	+
0	3+	4+	4+	2+	2+	3+	-	+	+	-	4+	2+	+	3+	3+	-	+	+
0	3+	4+	3+	2+	3+	3+	-	+	+	+	4+	4+	-	4+	3+	-	+	+
0	-	4+	3+	-	2+	2+	-	+	+	-	4+	3+	+	3+	2+	-	-	+
1	-	4+	4+	+	3+	3+	-	+	+	+	4+	4+	-	2+	2+	-	-	-
2	3+	4+	3+	-	3+	2+	-	+	+	-	4+	3+	+	3+	2+	-	-	-
3	3+	3+	3+	-	2+	2+	-	+	+	-	4+	3+	+	2+	2+	-	-	-
**4	-	+	+	-	-	+	-	-	-	-	2+	+	-	+	+	-	-	-
** 5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
** 6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
** 7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
** 9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
** 10												B I R D		D I E D				
**12		-	-		-	-												
**16		-	-		-	-												
19		+	+		-	-												
23		3+	3+		+	+												
26		3+	2+		+	+												
30		3+	3+		2+	2+												
33		3+	3+		3+	3+												

* Chicks 8 weeks old when experiment was started

** Chicks showing respiratory symptoms

TABLE V.

Degree of agglutination of erythrocytes, by plate method,

from chicks* inoculated with N1 strain of Newcastle disease virus

Chick Number / Virus Strain / Conc. Formalin in % — Days after inoculation	PC47B									PC48A								
	B1			N1			N2			B1			N1			N2		
	.05	.10	.15	.05	.10	.15	.05	.10	.15	.05	.10	.15	.05	.10	.15	.05	.10	.15
0	+	4+	3+	+	3+	3+	-	+	+	-	4+	3+	-	3+	3+	-	+	+
0	+	4+	3+	+	2+	2+	-	+	+	+	4+	3+	+	2+	2+	-	+	+
0	-	3+	2+	-	2+	+	-	-	+	+	3+	3+	-	+	+	-	-	-
0	+	3+	2+	+	2+	2+	-	-	+	+	4+	2+	+	2+	2+	-	-	-
1	+	4+	2+	+	3+	2+	-	+	+	+	4+	2+	-	+	2+	-	-	-
2	+	4+	2+	+	2+	2+	-	+	-	+	4+	3+	+	2+	2+	-	-	-
3	+	3+	2+	+	2+	2+	-	-	-	+	4+	2+	-	+	2+	-	-	-
**4	+	4+	3+	+	2+	2+	-	-	-	-	2+	+	-	-	+	-	-	-
** 5	-	3+	2+	+	2+	2+	-	-	-	-	+	-	-	-	-	-	-	-
** 6	+	3+	2+	+	2+	2+	-	-	-	-	+	-	-	-	-	-	-	-
** 7	+	4+	2+	+	3+	2+	-	-	-	+	4+	2+	-	+	+	-	-	-
** 9	-	2+	+	-	+	+	-	-	-	-	+	+	-	-	-	-	-	-
** 12		+	+		-	-					-	-		-	-			
** 16		+	+		-	-					-	-		-	-			
** 19		-	-		-	-					-	-		-	-			
** 23		-	-		-	-					-	-		-	-			
26		+	-		-	-					-	-		-	-			
30		3+	3+		-	-					3+	3+		-	-			
33		3+	3+		-	-					3+	3+		-	-			

* Chicks 8 weeks old when experiment was started

** Chicks showing respiratory symptoms

to return to normal (see Tables IV and V).

Experiment 2. Replicate of M1 virus strain From Table VI it may be seen that it is possible to duplicate some of the important results of the preceeding experiment. Disease symptoms and erythrocyte sensitivity change appeared the same day. Red blood cells from three of the four birds were insensitive to the M1 strain of Newcastle disease virus on the fifth day after inoculation and they remained so for a period of ten days to two weeks. On the ninth day after inoculation bird number PC430 succumbed to the disease. Table VI also demonstrates that M1 virus formalized with 0.15% formalin is not satisfactory to use as the viral agglutinating agent in the plate method, since erythrocytes from two of the birds were insensitive to it (non-agglutinable), before the birds were inoculated with the virus. The use of this particular strain treated with 0.15% formalin was continued throughout the plate method investigation, however, in order to have a comparison of the degree of agglutination of the B1 and M1 strains treated with the same concentration of formalin.

Experiment 3. Replicate of M1 virus strain With the use of whole blood, in place of a drop of citrated blood, it may be seen from Table VII that erythrocyte sensitivity began to diminish on the third day after inoculation. Symptoms of the disease, leg weakness and drooping wings as well as respiratory symptoms, appeared at this time. It is also interesting to note that on the third day after inoculation, erythrocytes from chick number 2PC432 were completely insensitive to the viral agglutinating agents, and that they remained so for a period of six days when the bird succumbed to the disease. It may be seen that by the ninth day after inoculation that two of the five birds had died from the disease, their blood having

TABLE VI.

Degree of agglutination of erythrocytes, by plate method,

from chicks* inoculated with N1 strain of Newcastle disease virus

DAYS AFTER INOCULATION	Chick Number / Virus Strains / Conc. Formalin in %	PC43C				PC46A				PC47C				PC 47D			
		B1		N1		B1		N1		B1		N1		B1		N1	
		.10	.15	.10	.15	.10	.15	.10	.15	.10	.15	.10	.15	.10	.15	.10	.15
0		4+	3+	3+	2+	3+	2+	2+	+	4+	2+	2+	+	4+	2+	2+	+
0		4+	3+	3+	2+	4+	+	+	-	4+	2+	3+	+	4+	-	+	-
1		4+	2+	3+	2+	3+	+	+	-	4+	2+	3+	+	3+	+	+	-
2		4+	3+	3+	2+	4+	2+	2+	+	4+	3+	3+	+	3+	+	+	-
3		4+	3+	2+	-	4+	+	2+	+	4+	+	+	-	3+	+	+	-
**4		4+	+	+	-	+	-	-	-	+	-	-	-	+	-	-	-
**5		3+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
** 6		3+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
** 7		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9		BIRD	DIED														
**11						-	-	-	-	-	-	-	-	-	-	-	-
** 14						-	-	-	-	-	-	-	-	-	-	-	-
** 18						-	-	-	-	+	+	-	-	-	-	-	-
21						+	+	-	-	+	2+	-	-	+	+	-	-
25						+	+	-	-	2+	2+	-	-	+	+	-	-
28						+	+	-	-	3+	3+	-	-	+	+	-	-

* Chicks 4 weeks old when experiment was started

** Chicks showing respiratory symptoms

TABLE VII.

Degree of agglutination of whole blood, by plate method,

from chick* inoculated with N1 strain of Newcastle disease virus

DAYS AFTER INOCULATION	Chick Number / Virus Strains / Conc. Formalin in %	2PC43E				2PC43F				2PC45K				2PC45M				2P C49F			
		B1		N1		B1		N1		B1		N1		B1		N1		B 1		N1	
		.10	.15	.10	.15	.10	.15	.10	.15	.10	.15	.10	.15	.10	.15	.10	.15	.10	.15	.10	.15
0		3+	+	3+	+	3+	+	3+	2+	3+	2+	3+	2+	4+	2+	4+	2+	+	-	+	-
0		3+	3+	+	-	4+	3+	2+	-	3+	2+	2+	+	4+	3+	2+	-	2+	+	-	-
2		4+	4+	2+	+	4+	4+	2+	+	4+	3+	2+	+	4+	4+	3+	+	3+	3+	+	-
**3		-	-	-	-	3+	2+	+	-	3+	3+	2+	+	2+	2+	+	-	2+	2+	-	-
**4		-	-	-	-	3+	2+	+	-	3+	3+	+	-	2+	2+	-	-	2+	2+	-	-
**5		-	-	-	-	2+	2+	-	-	3+	3+	+	-	+	+	-	-	+	+	-	-
**6		-	-	-	-	2+	2+	-	-	3+	3+	-	-	-	-	-	-	-	-	-	-
**7		-	-	-	-	+	+	-	-	+	+	-	-	-	-	-	-	BIRD		DIED	
**8		-	-	-	-	+	+	-	-	+	+	-	-	-	-	-	-				
** 9		Bird		Died		+	+	-	-	-	-	-	-	-	-	-	-				
**10						-	-	-	-	-	-	-	-	-	-	-	-				
**11						-	-	-	-	-	-	-	-	-	-	-	-				

* Chicks 4 weeks old when experiment was started

** Chicks showing symptoms of the disease

become non-agglutinable before death. In using whole blood it was most difficult to know whether to call some of the reactions marked +, positive or negative. Some were definitely positive while others were on the border line.

Experiment 4. B1 virus strain None of the chicks used in this experiment showed any symptoms of the disease or too much change in the degree of agglutination of their erythrocytes. Two explanations may be offered for this. B1 strain of Newcastle disease virus is known to be a very mild one. It has been used as a vaccine without showing any marked symptoms of the disease (12). Since these birds were removed to the Newcastle isolation building for a period of two weeks without showing any symptoms of the disease it is logical to assume that they became immunized without showing too much change in the degree of agglutination of their erythrocytes. It is also possible that during the period of isolation the erythrocytes may have become insensitive to the viral agglutinating agents, but soon regained their agglutinability. It may be seen from Table VIII that between the ninth and thirteenth day after inoculation that the degree of agglutination was about half that of the maximum or normal cells.

This experiment demonstrated the impractibility of using B1 virus strain as the viral agglutinating agent in the plate method. In two of the four birds used, cells from non-inoculated birds failed to agglutinate with the B1 virus strain treated with 0.10% and 0.15% formalin.

Experiment 5. Replicate of B1 virus strain Table IX demonstrates that B1 strain of Newcastle disease virus alters the red blood cells. On the tenth day after inoculation blood from all five of the birds used was completely insensitive to the viral agglutinating agents. It may be

TABLE VIII.

Degree of agglutination of erythrocytes, by plate method,

from chicks* inoculated with B1 strain of Newcastle disease virus

Chick Number / Virus Strains / Conc. Formalin in % / Days after inoculation	PC43B				PC45C				PC45D				PC47E			
	B1		N1		B1		N1		B1		N1		B1		N1	
	.10	.15	.10	.15	.10	.15	.10	.15	.10	.15	.10	.15	.10	.15	.10	.15
0	4+	3+	2+	+	4+	3+	2+	+	4+	2+	-	-	3+	2+	-	-
0	4+	3+	2+	+	4+	3+	2+	+	3+	+	-	-	3+	2+	-	-
2	4+	3+	2+	+	4+	3+	2+	+	3+	-	-	-	3+	+	-	-
3	3+	2+	2+	+	3+	3+	2+	+	3+	+	-	-	3+	2+	+	-
4	3+	2+	2+	+	3+	2+	2+	+	3+	+	-	-	2+	2+	+	-
5	4+	3+	2+	+	4+	3+	2+	+	3+	+	+	-	3+	+	+	-
6	3+	2+	2+	+	3+	2+	2+	+	3+	+	-	-	2+	+	-	-
9	2+	2+	+	+	2+	2+	+	+	2+	2+	+	+	2+	2+	+	+
13	2+	2+	-	-	2+	2+	-	-	2+	3+	-	-	+	+	-	-
16	2+	2+	-	-	3+	3+	-	-	3+	3+	+	-	2+	2+	+	-
19	3+	3+	-	-	3+	3+	-	-	3+	3+	-	-	2+	2+	-	-

* Chicks 6 weeks old when experiment was started

TABLE IX.

Degree of agglutination of whole blood, by plate method,

from chicks* inoculated with B1 strain of Newcastle disease virus

Chick Number / Virus Strains / Conc. Formalin in % / Days after inoculation	2PC45H				2PC45L				2PC46B				2PC48E				2PC49D			
	B1		N1		B1		N1		B1		N1		B1		N1		B1		N1	
	.10	.15	.10	.15	.10	.15	.10	.15	.10	.15	.10	.15	.10	.15	.10	.15	.10	.15	.10	.15
0	3+	+	3+	2+	4+	3+	4+	2+	3+	2+	3+	+	3+	+	3+	+	+	-	2+	+
0	3+	2+	+	-	4+	3+	2+	+	4+	4+	3+	+	3+	3+	2+	-	2+	+	-	-
2	4+	4+	-	-	4+	2+	4+	2+	4+	4+	3+	-	4+	4+	3+	+	3+	3+	+	-
3	3+	2+	+	-	4+	4+	3+	+	4+	4+	3+	-	4+	4+	3+	+	-	-	-	-
4	4+	4+	-	-	4+	4+	2+	+	4+	4+	2+	-	4+	4+	+	-	2+	2+	-	-
5	2+	2+	-	-	4+	4+	+	-	4+	4+	2+	-	3+	3+	-	-	2+	2+	-	-
**6	-	-	-	-	4+	4+	+	-	4+	4+	+	-	+	+	-	-	2+	2+	-	-
**7	-	-	-	-	+	+	-	-	2+	2+	-	-	-	-	-	-	-	-	-	-
**8	-	-	-	-	+	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-
**9	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
**10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
**11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* Chicks 4 weeks old when experiment was started

** Chicks showing symptoms of disease

seen that symptoms of the disease and erythrocyte agglutination change appeared on the sixth day after inoculation. In comparison with Table VII it may be seen that when chicks were inoculated with N1 strain the symptoms of the disease and degree of agglutination change appeared three days earlier, pointing out the fact that N1 strain of Newcastle disease virus affects chicks earlier than the B1 strain. It appears from this experiment that even a low virulent strain of Newcastle disease virus may alter h the erythrocytes in such a way that they are no longer agglutinable.

Experiment 6. NB virus strain Table X illustrates very clearly the virulence of the NB strain of Newcastle disease virus under investigation. All of the birds succumbed to the disease before their erythrocytes became entirely insensitive to the virus. Note, however, that in three of the four birds there was a change in the sensitivity of the erythrocytes before the birds died.

Comparison of tube agglutination and plate agglutination A decrease in cell titer is shown graphically (Figures 1-15) in all fifteen birds used in the tube agglutination method, but not with each virus strain employed in the test. A decrease in degree of agglutination is shown in a representative graph (Figure 16) of four birds used in the plate method. By comparing Figures 1-15 with Figure 16 one may see that there is a correlation of the agglutinating sensitivity of infected red cells between the tube and the plate method. The tube agglutination method appears to be the more sensitive of the two methods since sensitivity change appeared earlier and once sensitivity had reached a minimum it soon returned to normal (see Figures 1-15). In the plate agglutination method this minimum held for as long as fourteen days (see Figure 16).

TABLE X.

Degree of agglutination of erythrocytes, by plate method,

from chicks* inoculated with N2 strain of Newcastle disease virus

Chick Number / Virus Strains / Conc. Formalin in % / Days After Inoculation	PC43A				PC45E				PC47B				PC48B			
	B1		N1		B1		N1		B1		N1		B1		N1	
	.10	.15	.10	.15	.10	.15	.10	.15	.10	.15	.10	.15	.10	.15	.10	.15
0	3+	+	2+	+	4+	3+	3+	2+	4+	2+	3+	2+	4+	2+	2+	+
0	4+	+	2+	+	4+	3+	3+	2+	4+	2+	+	-	4+	+	+	-
1	3+	+	+	-	4+	3+	3+	2+	4+	2+	+	-	4+	+	+	-
2	4+	+	2+	-	4+	3+	4+	2+	4+	3+	2+	+	4+	+	+	-
3	3+	-	+	-	4+	3+	3+	2+	4+	+	2+	+	4+	+	+	-
** 4	2+	-	-	-	4+	3+	+	+	2+	+	+	-	2+	+	-	-
** 5	2+	-	-	-	4+	3+	-	-	2+	+	-	-	2+	+	-	-
** 6	+	-	-	-	4+	2+	+	-	BIRD		DIED		BIRD		DIED	
7	BIRD		DIED		BIRD		DIED									

* Chicks 4 weeks old when experiment was started

** Chicks paralyzed

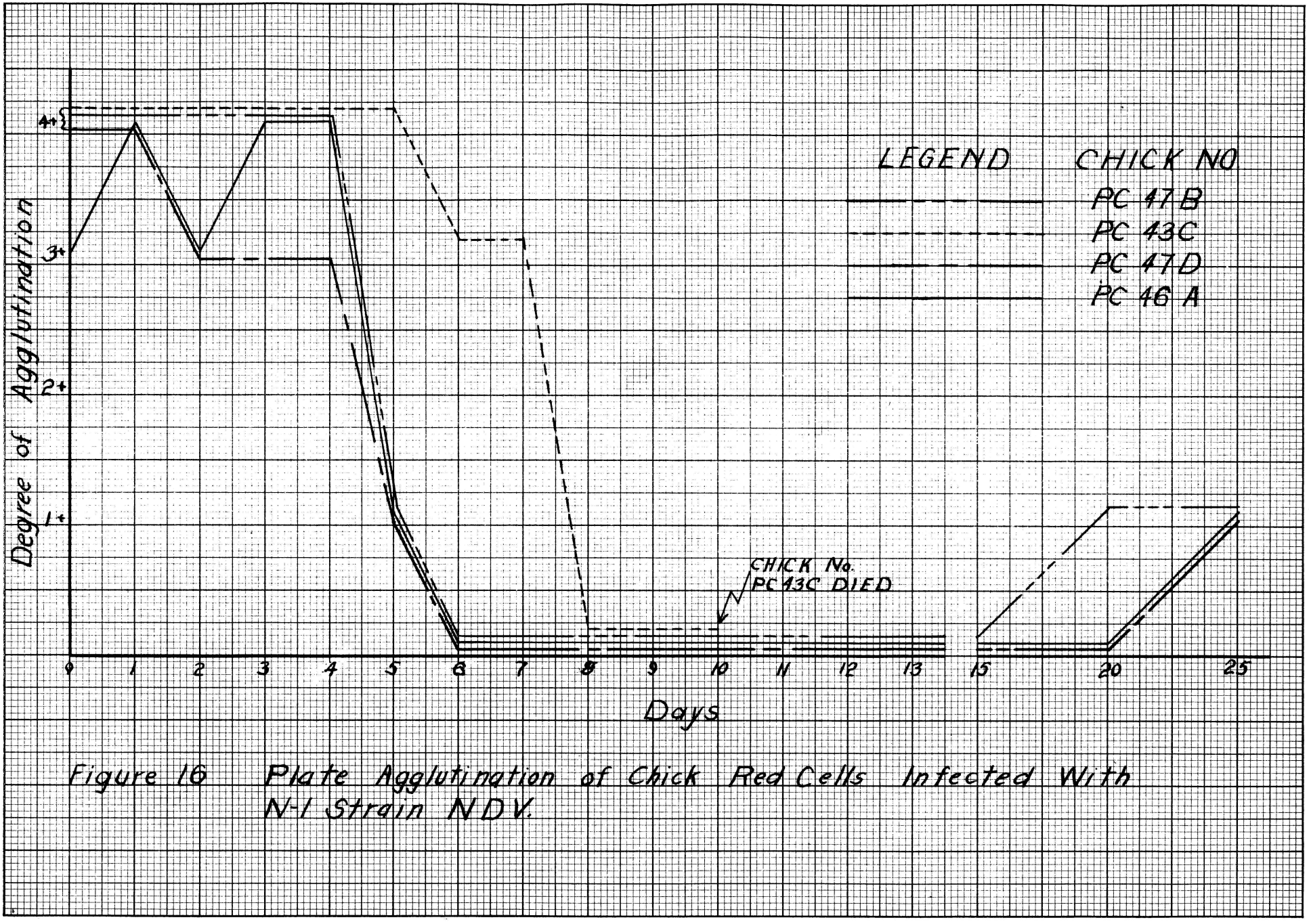

Figure 16 Plate Agglutination of Chick Red Cells Infected With N-1 Strain NDV.

CONCLUSIONS

1. Normal erythrocytes from individual chickens vary in their agglutinability with the virus strains of Newcastle disease investigated. The erythrocytes of different chickens may range from low to high in their sensitivity to Newcastle virus hemagglutination. Strains of viruses also vary in their ability to agglutinate red cells.

2. Once the normal erythrocyte comes in contact with either B1, M1, or M2 strain of Newcastle disease virus it begins to lose sensitivity for the virus.

3. Erythrocytes from infected chicks start to lose sensitivity for Newcastle virus around the fourth day after inoculation, as demonstrated in the rapid-plate technique. The cells become insensitive around the ninth or tenth day after inoculation and remain so for as long as two weeks.

4. All erythrocytes from non-infected chicks used in the plate method were sensitive to the B1 strain of virus used. Most were sensitive, in some degree, to M1 and M2 strains of the virus. But with the use of B1 strain, a sharper demarcation between negative and positive agglutination was obtained.

SUMMARY

An *in vivo* study of the alteration of the erythrocytes from forty-six young chickens of various breeds infected with three strains of Newcastle disease virus has been made.

Three strains of Newcastle disease virus which varied in their pathogenicity for chickens, namely B1, M1, and M2 strains, have been studied, comparing the agglutinability of the red blood cells by the tube hemagglutination method and the rapid-plate technique.

Graphs and tables have been prepared showing the *in vivo* effect of the virus strains on the chicken erythrocytes when the viral strains are used as the agglutinating agent.

By mixing a drop of whole blood with 0.05 ml. of the B1 strain of Newcastle disease virus formalized with 0.10% formalin it may be possible to diagnose Newcastle disease in chickens, without the aid of the standard hemagglutination-inhibition (HI) test.

The author believes that with more study the plate method may be of real value as a screening or presumptive test in the diagnosis of Newcastle disease infection.

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