THERMAL INACTIVATION OF NEWCASTLE DISEASE VIRUS

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This is to certify that the

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THERMAL INACTIVATION
OF NEWCASTLE DISEASE VIRUS

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Table of contents

Object

The object of this work was to study the effect of various temperatures on the infectivity and on the hemagglutinating activity of Newcastle disease virus.

REVIEW OF LITERATURE

Infectivity

Doyle[15], in 1927, first demonstrated that Newcastle disease (avian pneumoencephalitis)[3] was due to a filtrable virus. He reported that at 37°C the virus in infected, triturated organs was active after 48 hours but inactive after 72 hours. In 50 per cent glycerine in physiological saline the virus was active after 197 days but inactive after 259 days. In physiological saline the virus was active at 86 days but inactive at 100 days. Virulent spleen was active after 80 days but inactive after 150 days. Blood in a sealed pipette was active after 109 days. Ground liver, lung, spleen and brain, dried in a desiccator over $CaCl_2$ in the cold, was active at 40 days but not at 100 days. Kidney under the same conditions was active at both 40 and 100 days but the ovary was inactive at both time intervals. Doyle[16] showed that bone marrow and muscles of chilled carcasses were infective after six months.

Farinas[17] demonstrated that 2cc samples of suspensions of virus-infected organs in thin glass vials at 75°C, 62°C, and 55°C were rendered innocuous in 30 minutes. Samples at 50°C, 42°C, and 37°C were viable at 30 minutes and samples at ice temperature were viable for several months. Virus in diluted crop content was inactivated at 37°C in three days. Virulent crop content dried on cotton was inactivated in five days at room temperature but liquid crop content remained virulent for 15 days. A Berkefeld "W" filtrate remained virulent after an exposure of one day. Ground liver, lung, spleen and brain dried in a desiccator over $CaCl_2$ in the cold, was active for five months and 18 days. Heart and spleen in pure glycerine stored at 0°C - 2°C remained infective for 175 days. Dilute crop content placed in Petri dishes which were floated on water to prevent overheating were inactivated in 30 minutes when exposed to the sun in the Philippine Islands from 10 A.M. to 1 P.M. Liquid crop content in distilled water stored at ice-chest temperature (0°C - 4°C) remained virulent for 5 months and 11 days but organ suspensions in distilled water were inactivated in two months and 18 days. Crop content in a 50 per cent aqueous solution of glycerine was innocuous at four months and 20 days under the same conditions but crop content in 50 per cent physiological saline solution and 33 1/3 per cent glycerine was inactivated in two months and 26 days. Berkefeld "W" filtrate of crop content in distilled water stored under the same conditions was virulent for one month and 22 days. Dilute crop content in distilled water plus 30 per cent Sorensen's phosphate buffer with a final pH of 7.2 stored at ice-chest temperature (0°C - 4°C) was virulent for 320 days.

Iyer [30] reported that heating to 50°C or higher inactivated the virus in organ suspensions. Liver and spleen were infective after being stored in the refrigerator for about six months.

When desiccated *in vacuo* over P_2O_5 and stored in the refrigerator they remained virulent for about three years. Exposure of a saline suspension of virus-infected organs to direct sunlight for an hour had no effect on the virus.

Burnet[10] showed that Newcastle disease virus in the form of infected extraembryonic fluids was moderately resistant to heat. After 15 minutes at 60°C the virus titer was about $1/10^8$ of the original as determined by pock-counting titration method but the infectivity was destroyed at 65°C in 15 minutes.

Brandly *et al*[7] reported that colorless, clear, infected-allanto-amnionic fluid in the lyophilized state was inactivated after 30 minutes at 60°C or within 45 minutes at 55°C. High dilutions of infected-allanto-amnionic fluids in broth or saline retained their infective titers for 24 hours or longer at room temperature (22°C - 25°C). The same materials retained their titers for a week in the refrigerator at 6°C - 8°C. The infectivity of allanto-amnionic fluids either in the moist or lyophilized state was unaltered after 23 months storage at 0°C- to -70°C, the longest period tested. Virulent allanto-amnionic fluid in 35 ml. quantities in large quartz tubes was inactivated in 35 to 55 minutes when exposed to rays of 2537 Å. Exposure of the supernatants of infected allanto-amnionic fluid-embryo pulp mixtures to rays of 1600 - 1800 Å in flat quartz chambers of the ultraviolet irradiation apparatus of Oppenheimer and Levinson and associates inactivated the virus in from 0.8 to 1.08 seconds.

Picard [39] reported that brain dried for 48 hours at 27° Celsius and stored over $CaCl_2$ remained infective for 35 days. Twenty-four hours exposure to daylight of a 1:10 dilution of saliva in a cotton-plugged tube inactivated the virus.

Haddow [20] showed that dried vaccine was virulent after about two years in the cold.

Hudson [29] reported that brain, spleen and liver in 50 per cent glycerine stored at ± 1°C were infective for 239 days, the longest period tested.

Beach[2] demonstrated that virulent desiccated lungs stored in the refrigerator remained infective for 195 days. Tissues containing active virus preserved in 50 per cent glycerine remained fully active for 50 days but the virulence appreciably decreased in 85 days. In sealed vials in a dry-ice refrigerator these tissues remained fully active for seven months, the longest period tested.

Hemagglutinative activity

Hirst [23], in 1941, noted that when the allantoic fluid from chicken embryos infected with the PR8 and Lee strain of influenza A virus was being collected, the red blood cells from ruptured vessels were agglutinated in the allantoic fluid. Red cells in the allantoic fluid of chicken embryos inoculated with sterile materials did not agglutinate. Hirst demonstrated that this hemagglutination phenomenon was the result of infection of the embryonating chicken eggs with the PR8 and Lee strains of influenza. Hirst [24] later showed that the addition of specific immune serum inhibited the hemagglutination in the presence of the homologous virus, but not when the heterologous strains were used.

McClelland and Hare [34] independently reported the prompt agglutination of human, guinea-pig, hen and chick red blood cells when mixed with allantoic fluid containing either influenza A or B virus. They found that the red cells of rabbit, rat, mouse, horse and sheep would not agglutinate.

Hirst [24] and McClelland and Hare [34] clearly demonstrated that the agglutination of chicken erythrocytes in the presence of influenza virus was a valuable basis for the quantitative determination of hemagglutinative activity of influenza virus.

In the original Hirst test [24] the density of the red blood cell suspensions was compared with the density of known standard

suspensions of red cells in saline to make the grading of the reaction more objective. Hirst employed the amount of red cell sedimentation as reflected by clearing of the supernatant as an index of the degree of the reaction rather than visible hemagglutination. He demonstrated that the infectivity of the virus could be destroyed without destroying the hemagglutinative activity of the virus. The infectivity of the allantoic fluid preparations from embryonating chicken eggs was completely destroyed at 56°C for 15 minutes, while the hemagglutination titer remained constant. Storage of the infected allantoic fluid at room temperature for several days resulted in a slowly diminishing titer for mice but the hemagglutination titer remained constant. He also pointed out that the hemagglutination titer of the infected allantoic fluid and its virulence for mice were relatively constant regardless of the influenza strain used. The fact that eggs inoculated with different strains of virus yielded allantoic fluids of approximately the same hemagglutination titer suggested that the virus particle concentration in allantoic fluid infected with different strains of influenza was approximately the same and that their capacity to agglutinate red cells was also nearly identical for individual virus particles. Based on this assumption, Hirst pointed out that the test provided a simple method for determining the relative number of virus particles in suspensions independently of virulence.

Later, Burnet[9] and other investigators[37] used variations of this procedure for titration of hemagglutinative activity of influenza virus. They used the pattern of the agglutinated cells as a basis for reading the test.

In 1942, Hirst and Pickels[25] described a photoelectric densitometer for a more accurate and reproducible measurement of hemagglutinative activity of virus preparations.

Hirst[26] reported that, when suspensions of PR8 and Lee strains of influenza virus were heated at 55°C for an hour, about half of the hemagglutinating activity was lost, while at 50°C for the same length of time there was no detectable effect on the hemagglutination titer of the suspensions. At 60°C the hemagglutinating activity of both strains were rapidly inactivated.

In 1943, Stuart-Harris[44] demonstrated that the use of red blood cells from different chickens or even the cells of the same bird at different times of the year gave significant differences in hemagglutination-inhibition tests with influenza virus.

This variation in titer due to the use of cells from different chickens was confirmed by Hirst[27]. Using the PR8 strain of virus in hemagglutination tests and the homologous antiserum in hemagglutination-inhibition tests he found an approximate two-fold variation in end points when cells from different donors were used. The Lee strain of virus and its antiserum gave similar results. He also found variations in titer as the result of aging of the red blood cells

at 4°C and on standing for a few hours at room temperature.

Miller and Stanley[35] tested the influence of hydrogen ion concentration on the hemagglutination titer of influenza virus. They found that the range of pH 6 to 8 had only a negligible effect but at pH 9 a lower titer was obtained. They also concluded that phosphate buffer diluent offered no great advantage over 0.85 per cent sodium chloride solution. They obtained the best results when the ingredients were incubated at 24°C (room temperature) and they found that red cells from different chickens showed marked variability in hemagglutination titers. In a series of experiments on the effect of red cells stored for various lengths of time on the chicken cell agglutination test (CCA), some preparations showed a slight decrease in hemagglutinability in two days at 4°C with a considerable loss of hemagglutinability at the end of eight days, whereas others were stable from five to nine days. In other experiments they found that a 50 per cent loss in hemagglutinative property occurred in seven days. They also pointed out that storage of packed red cells did not contribute to their stability to an appreciable degree. The CCA activity of influenza virus was more stable at 4°C than at -70°C.

Miller[36] demonstrated that the infectivity and CCA activity of different strains of influenza virus were more resistant to an alkaline environment than to an acid environment. The rate of decrease in infectivity was greater than the rate of decrease in CCA activity. The virus was more stable at 4°C than at 23°C.

Twyble[46] used PR8 influenza virus-infected allantoic fluid preparations and obtained higher hemagglutination titers with chicken and duck cells than with pigeon and human cells. He also pointed out that the infectivity and hemagglutinative activity of the virus are closely associated and could not be separated by filtration.

In 1944, Salk[41] described a simple method for titrating the hemagglutinating capacity of influenza virus and the corresponding anti-body. He used a suspension of 0.25 per cent of red cells made from a 10 per cent stock preparation stored at about 4°C. He pointed out that the cells could be used more than a week if stored under sterile conditions. In complete agglutination (+) the clumps of cells settled to the bottom of the tube with a dispersed pattern rather than an aggregated pattern. A negative pattern (0) consisted of a central, sharply demarcated disc. Irregular clumps of cells associated with a halo of finely aggregated or unagglutinated cells represented a reaction intermediate (±) between complete hemagglutination and no hemagglutination. The highest dilution of the virus producing maximal agglutination of red cells represented the end point and the titer was expressed as the reciprocal of the final dilution of virus after the red cell suspension was added.

Burnet[9] found that the virus of Newcastle disease of chickens agglutinated chicken red blood cells in a manner similar to that shown by influenza virus strains.

Lush[33] found that the Hirst method of reading the hemagglutination test based on the reduction of opacity of the supernatant fluid as a result of red cell sedimentation was not applicable to Newcastle disease virus because the virus affected only a small proportion of the cells and did not reduce the density of the suspension sufficiently to permit the use of the Hirst method. Therefore, reading of the test by the pattern of the agglutinated cells was necessary. Lush made a graded recording of the reaction as ++++, +++, ++, +, ± and 0, representing maximal agglutination to no agglutination with intermediate gradation. He used a two per cent suspension of red cells and the readings were made after two hours.

Clark and Nagler[12] and Burnet[9] demonstrated differences in agglutinative activity of Newcastle disease virus with red cells of various species of animals.

Burnet[9] and Lush[33] used, respectively, an Australian and European strains of Newcastle disease virus. When the immunologic relationship between avian pneumoencephalitis of the United States of America and the classical Newcastle virus of other parts of the world was demonstrated[3, 6] studies were made on the red cell agglutinating ability of avian pneumoencephalitis virus.

Beach demonstrated that avian pneumoencephalitis virus could cause agglutination of red blood cells of chickens and the results were given verbally[4] at the 1944 Conference of Research Workers in North America. Beach[4] reported that the phenomenon of red blood cell agglutination could be applied to avian pneumoencephalitis virus. The patterns of the sedimented cells were taken as the criterion for reading the test instead of changes in the intensity of color of the supernatant fluid as was done by Hirst[24]. The highest dilution of allanto-amnionic fluid which gave at least a ++ agglutination of red cells (a small central disc surrounded by a granular area of agglutinated cells) was termed the hemagglutination titer of the virus.

Burnet et al[10] showed that there was a sharp dissociation between destruction of infectivity and of hemagglutinative activity of Newcastle disease virus. He observed that after 15 minutes at 60°C the infectivity titer was about $1/10^8$ of the original titer as determined by pock-counting titration method, but the hemagglutination titer was still 50 per cent of the original titer. The hemagglutinating activity was completely destroyed at 65°C in 15 minutes. He commented that particles which were smaller or were less readily destroyed than the virus particles might be responsible for a small but definite portion of hemagglutinating activity. This comment was made after some experiments on dissociation of infectivity and hemagglutination with Seitz filtration of the virus material.

Brandly et al[7] obtained results different from those of Lush[33] when they used the Hirst phenomenon to demonstrate

Newcastle virus in infected allanto-amnionic fluids. Satisfactory results were obtained when the red cell suspension was reduced from two to one per cent or less and when the tubes were incubated at room temperature rather than at ice box temperature with readings made at 15, 30 and 45 minutes instead of at two hours. Thirty minutes at 60°C, 45 minutes at 55°C, ultraviolet irradiation at 2537 Å for 45 to 55 minutes and at 1600-1800 Å for 0.8 to 1.08 seconds were sufficient to destroy the infectivity but were without effect on the hemagglutinative activity of the virus.

Friedland and Pickels[19] demonstrated by data obtained from centrifugation experiments that the sedimentation rates of the infectious principle and of the hemagglutinative activity of influenza virus were roughly the same. Particles of at least 60 millimicrons in diameter were associated with both activities.

Later, Stanley[43] showed that influenza virus particles, having a diameter of 115 millimicrons, had an hemagglutinating activity and infectivity higher than the smaller particles.

From the work of Sharp et al[42], Taylor et al[45], Stanley[43], Lauffer and Miller[32], Miller, Lauffer and Stanley[37] and Knight[31], facts were obtained leading to the conclusion that the infectivity in influenza virus is closely associated with the red cell agglutinating factor and that spheres of 115 millimicrons in diameter were responsible for both properties.

Cunha et al[14] reported that the hemagglutinative capacity of Newcastle disease virus was always closely associated with either the infective particles as indicated by infectivity measurement, or with a material consisting of sperm-shaped particles, closely resembling T_2 bacteriophage as seen under the electron microscope.[28]

Florman[18] reported that hemagglutination by Newcastle disease virus was markedly affected by temperature. At room temperature the reaction was difficult to read, especially with low dilution of virus. The test was easily read and end points were readily determined by incubation at 4°C at which temperature the virus was more completely adsorbed by and less rapidly eluted from chicken red cells.

Hanson et al[21] studied the effect of the route of Newcastle disease virus inoculation on embryo lethal titers as well as on hemagglutination titers. Inoculation of the virus by the intravenous route caused the most rapid development of hemagglutinating activity. The embryo LD_{50} titration was more sensitive in detecting small quantities of virus than the hemagglutination test. It was possible to detect the virus by embryo infectivity tests as early as six hours following inoculation by the intravenous route, but the hemagglutination test was first found positive 18 hours after inoculation by the same route.

Hanson et al[22] reported that for the several Newcastle disease virus strains studied there was a marked variation in the correlation of the hemagglutinating activity and the infectivity for embryonating eggs.

Winslow[48] found that prolonged serial transfer of two attenuated strains of Newcastle disease virus resulted in no change in their hemagglutinating activity.

Brandly et al[8] reported that storage of allanto-amnionic fluid infected with Newcastle disease virus in the frozen state for several months did not result in appreciable loss of hemagglutination titer. They confirmed the variations in sensitivity among the cells of different birds to virus agglutination.

Burnet and Anderson[11] reported that red cells treated with amnionic and allantoic fluid preparations of Newcastle disease virus develop a new antigenic character which allow them to be agglutinated to high titer either by experimental Newcastle disease immune serum or by sera from cases of infectious mononucleosis in man. They claimed that an agent (cell sensitizing agent or CSA) other than the virus propagated in chicken embryo is responsible for this changed character of the cells by adsorption to their surface.

Anderson[1] showed that cells thus sensitized as reported above by Burnet[11] were able to agglutinate untreated fresh chicken red cells and that the agglutinating principle on these stable cells could be titrated by a method similar to that used for titration of the

hemagglutinating activity of the virus. He demonstrated that the CSA was a virus particle which remains attached to the cell after the virus aggregate leaves the cell; that the cells coated with CSA, thoroughly washed, retained their infectivity for chicken embryos, and that its presence on the red cell rendered the cell susceptible to agglutination by Newcastle disease virus immune serum.

Burnet et al[10] had indicated a possible distinction between hemagglutinating activity and infectivity in Newcastle disease virus particles. Small particles were able to cause hemagglutination but they were relatively non-infective. Anderson[1] showed that these particles were single elements from a virus aggregate whose infective power was reduced to some degree as result of separation from the virus aggregate.

MATERIALS AND METHODS

Virus.

A strain of Newcastle disease virus (48-9-328) isolated in the Department of Bacteriology and Public Health, Michigan State College, on November 9, 1948 from a naturally infected chicken was used as inoculum. The virus had been originally established and cultivated in the allantoic cavity of embryonating chicken eggs through five passages and was capable of killing all inoculated embryos by the end of the second day following inoculation.

White Leghorn eggs obtained from a commercial hatchery were used in all studies. The embryos were from 9 to 11 days old but in each test all embryos employed were of the same age. The eggs were incubated at 99°F in an electric, forced-draft incubator.

The virus suspension used in the studies consisted of pooled allantoic fluid from embryos dead on the second day following inoculation. Sixty 10-day old embryos were inoculated via the allantoic cavity. The eggs were transilluminated for selection of an area of the chorioallantoic membrane, free from large blood vessels about 3 mm. below the base of the air cell. A small hole was drilled through the shell by means of a small drill attached to the chuck of an electric motor without piercing the shell membrane. Another hold was drilled through the shell over the top of the air cell. Before inoculation, tincture of metaphen was painted over the holes and allowed to dry. The shell membrane over the air cell was punctured with a sterile dissecting needle to allow equalization of pressure within the egg when the inoculum was injected into the allantoic cavity and to prevent leakage of the inoculum from the site of injection.

The eggs were inoculated via the allantoic cavity with a B-D Yale, 1 cc. capacity tuberculin syringe fitted with a 27-gauge, 1/2 inch needle. The needle was inserted through the hole on the side of the egg, piercing the shell membrane and underlying chorioallantois for a few millimeters, and the inoculum was then injected. The holes in the shell were sealed with melted paraffin and the eggs returned to the incubator.

All the eggs were candled before the first 24 hours after inoculation to discard any embryos as embryo mortality before the first 24 hours was considered to be due to non-specific causes and not to the action of the virus.

In subsequent candlings the dead embryos were removed from the incubator and chilled in the refrigerator at 4°C overnight to diminish possible hemorrhage from ruptured vessels when the allantoic fluid was collected. A small batch of the eggs was removed at a time from the refrigerator for collection of the infected allantoic fluid. The shell over the air cell was swabbed with tincture of metaphen, allowed to dry, and the shell was cracked and removed with sterile forceps. Sterile 5 or 10 cc syringes fitted

with 20-gauge, 1 inch needles, were employed for collecting the allantoic fluid. The needle was inserted into the allantoic sac and the fluid was aspirated into the syringe and expelled into sterile, screw cap, 8 ounce prescription bottles. All fluids were bacteriologically sterile.

The fluid was frozen for three days at -45°C, thawed at room temperature and dispensed into 50 ml. Lusteroid tubes with rubber caps previously sterilized by exposure to ultraviolet light rays of 2537 Å wave length. The tubes were centrifuged for 20 minutes in an angle head in an International PR-1 centrifuge at 38-40°F at 4,400 r.p.m. (approximately 2780 G). The supernatant was distributed in sterile screw cap vials in amounts sufficient for the particular experiment in which it was to be used. The vials were then stored at - 45°C until needed.

Titration of virus infectivity.

The same procedure for titrating virus infectivity was used throughout the experiment. Serial tenfold dilutions of the virus-infected allantoic fluid were made in Difco nutrient broth. Eleven tubes (13 X 100 mm.) each containing 4.5 ml. of broth were placed in a rack and 0.5 ml. of virus was added to the first tube with a 1 ml. serological pipette. The contents of the tube were mixed by aspirating the mixture into and expelling it from a 2 ml. serological pipette 20 times. Using the same pipette, 0.5

ml. of this mixture was transferred to the second tube and the pipette was then discarded. Another pipette was used to mix the contents of the second tube and transfer to the third tube. This process was continued until all dilutions were made.

Five eggs per dilution were inoculated with 0.1 ml. per egg via the allantoic cavity starting with the highest virus dilution and progressing to the lowest dilution using one syringe for the entire operation.

Bacterial sterility was checked by incubating the dilution tubes at 37°C for 48 hours.

The eggs were candled daily and the mortality recorded. Embryos dead within the first 24 hours following inoculation were not included in the final mortality rates.

Quantitative determination of viral activity was calculated according to the 50 per cent end-point (LD_{50}) method of Reed and Muench[39]. According to this formula the titer of the virus was the highest dilution in which 50 per cent or more of the embryos were killed by the virus. The number of embryo lethal doses of virus in the undiluted inoculum were expressed as the reciprocal of the logarithm of the titer of the virus. The titer of the virus suspension used throughout the experiment was $10^{-9.32}$ and there were considered to be $10^{9.32}$ embryo lethal doses per 0.1 ml. of the undiluted inoculum.

Titration of virus hemagglutinating activity.

Samples were also titrated for their hemagglutinating activity for red blood cells from chickens.

Materials.

Virus. The virus preparation consisted of blood-free, pooled allantoic fluid from embryonating chicken eggs infected with Newcastle disease virus.

Saline solution. Chemically pure sodium chloride (0.85%) in distilled water.

Red blood cells. Blood from one chicken was used throughout the experiment in an endeavor to reduce variations in hemagglutination titers obtained with cells from different donors[8, 26, 34, 43]. The blood was collected in a chemically clean, sterile tube containing 1 ml. of 2 per cent sodium citrate in saline[46] per 9 ml. of blood. The blood was mixed thoroughly to prevent clotting. The red blood cells were sedimented by centrifugation at 1600 r.p.m. for 8 minutes and the plasma was removed. The cells were then washed three times by centrifugation with quantities of saline equal to ten times the volume of packed cells. In the washing process the first two cycles of centrifugation were at 1600 r.p.m. for 8 minutes and the last centrifugation was at 1000 r.p.m. for 10 minutes. The supernatant was then removed and the packed cells were stored in the refrigerator at 4°C. Cells were found to be satisfactory for use in the test for as long as six days[26, 46]. A 0.5 per cent saline suspension of the cells was prepared immediately prior to use in the test.

Procedure.

The tubes used in the test were chemically clean, round bottom tubes (75 mm. X 10 mm.). Serial two-fold dilutions of the virus from 1 in 5 through 1 in 5120 were made in saline solution in a row of tubes in a wire rack. Transfers of 0.25 ml. of each virus dilution were made into a parallel row of tubes and 0.25 ml. of saline was added to each tube. The tubes were shaken well and 0.25 ml. of a 0.5 per cent suspension of red blood cells was added to each tube. The control tube contained 0.5 ml. of saline and 0.25 ml. of cells. The tubes were immediately shaken to mix the cells and were then incubated at room temperature (22°C- 27°C) for one hour. A protocol of the procedure used in titrating the hemagglutinating activity of the materials is given in Table I.

The test was read by placing the rack of tubes under a fluorescent light and viewing the pattern of the cells in the bottom of the tubes with the aid of a mirror under the rack.

Maximum hemagglutination was exhibited as a uniform layer of agglutinated cells covering the bottom of the tube and was recorded as positive (+). In the control tube and in the tubes where there was no agglutination, the sedimented cells formed a central, sharply demarcated red disc in the bottom of the tube. This reaction was designated as negative(-). Intermediate reactions between maximum agglutination and no agglutination were observed in which the pattern of the normally sedimented cells of a negative test was surrounded by a zone of agglutinated cells. This pattern was designated as partial hemagglutination (±).

The hemagglutination titer of the virus was considered to be the highest dilution of the virus, before the addition of saline and cells, in which maximum hemagglutination was present. Twenty-five hundredths ml. of that particular dilution was considered to contain one Hemagglutinating Unit (HU). The number of Hemagglutinating Units in 0.25 ml. of the undiluted virus was considered to be the reciprocal of the titer of the virus.

The result of this titration is shown in Table II.

EXPERIMENTAL PROCEDURE

Thermal inactivation of the virus was evaluated on three bases: qualitative and quantitative infectivity tests with embryonating chicken eggs and hemagglutination tests.

The qualitative infectivity tests were principally used for the room temperature (22°C- 27°C), 37°C, 56°C, 62°C, 77°C and 100°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 0.1 cc. of the respective virus samples into ten 10-day embryonating chicken eggs via the allantoic cavity. The criterion for inactivation of the virus was failure of the virus to kill the embryo.

The procedure for the quantitative infectivity tests and hemagglutination tests have been previously described.

For the 4°C study, 25 ml. of the virus was placed in a 30 ml. screw cap vial (95 X 25 mm.) and stored in a previously chilled beaker of water in the refrigerator. When the vial was removed from the refrigerator for sampling of the virus, it was placed in a THERMOS laboratory vessel containing water at 4°C. When the vial was returned to the refrigerator it was placed in the beaker of water. At certain predetermined time intervals a sample of the virus was removed from the vial. One portion of the sample was immediately tested for qualitative and quantitative infectivity and the other portion was placed in a 4 ml. screw cap vial and stored at - 45°C for subsequent hemagglutination tests. All hemagglutination tests for this study were made with the same preparation of red blood cells to reduce any error attendant upon variables which might occur with different preparations of red blood cells[8, 26, 34, 43].

Samples of the virus for the room temperature (22°C -27°C) and 37°C studies were distributed in 2.5 ml. amounts in 5 ml. thin wall glass ampoules and the ampoules sealed. The room temperature samples were submerged in a water bath which was placed on a shelf in the laboratory. Frequent temperature readings showed a variation from 22°C to 27°C during the test period. The 37°C samples were submerged in electric, thermostatically controlled water bath. At 24-hour intervals a sample of virus was removed from each water bath and stored at - 45°C for subsequent qualitative and quantitative tests and hemagglutination tests. When the ampoule was opened for removal of a sample for qualitative infectivity tests, the remainder of the sample was placed in a 4 ml. screw cap vial and stored at - 45°C for subsequent quantitative infectivity tests and hemagglutination tests.

Samples of the virus for the 56°C, 62°C and 77°C studies were distributed in 2.5 ml. amounts in tubes (75 X 10 mm.) and placed in racks in electric, thermostatically controlled water baths with a motor driven agitator to insure equalization of temperature

throughout the bath. At certain predetermined time intervals of minutes, samples of the virus were removed from the water baths and immediately chilled in crushed ice in a THERMOS laboratory vessel. Embryos were injected for qualitative infectivity tests. The remainder of the sample was then stored in 4 ml. screw cap vials at - 45°C for subsequent quantitative infectivity tests and hemagglutination tests. Previous experience with erratic zone-like results in inactivation tests had shown that it was necessary to avoid allowing any virus to adhere to the wall of the tube above the water level in the bath. Virus above the water level would not be subjected to the inactivation temperature and when the sample was removed for testing the remaining active virus on the wall of the tube would be incorporated with the inactive virus and produce an inaccurate protocol.

Samples of the virus for the 100°C study were processed in the same manner as those for the 56°C, 62°C and 77°C studies with the exception that the tubes were placed in a beaker of water which was boiled over a Bunsen burner.

RESULTS AND DISCUSSION

Quantitative infectivity tests of the virus exposed at 4°C refrigeration temperature showed a progressive decrease in titer from $10^{-9.32}$ to $10^{-7.62}$ during the 13 weeks of exposure. The increase in titer from $10^{-8.50}$ to 10^{-9} at the 4th week and from $10^{-8.31}$ to $10^{-8.37}$ at the 9th week could not be explained on the basis of increase in virulence of the virus. These differences might possibly be due to the death of a few embryos from causes other than the virus. See Table III.

In contrast to the decrease in titer of quantitative infectivity tests, the hemagglutinating activity of the virus at 4°C remained constant through the 13 weeks, the longest period tested. See Table IV.

Mortality of all inoculated eggs occurred from the first day through the 13th day in qualitative infectivity tests of the virus exposed at room temperature (22°C- 27°C). The mortality rate gradually decreased from 90 per cent at the 22nd day to 30 per cent at the 30th day. See Table V.

In the study of the quantitative infectivity of the virus exposed at room temperature, a progressive decrease in titer from $10^{-9.32}$ to 10^{0} was seen during the 30 days of exposure. On the 1st day the titer was $10^{-7.83}$ and it remained constant through the 3rd day. At the 5th day the titer was $10^{-6.37}$ and it continued to

decrease progressively through the 30th day with exceptions at the 7th, 14th and 17th days where the titer was higher than at the previous test periods. These differences might possibly be explained on the same basis mentioned above. See Table V.

The hemagglutination titer decreased from + at 1280 to ± at 1280 at the end of the 1st day and it remained constant through the 19th day. The titer was + at 640 at the 21st day and it remained constant through the 30th day. See Table VI.

Qualitative infectivity tests of the virus at 37°C showed a mortality of all inoculated eggs through the 3rd day. The mortality rate decreased to 50 per cent on the 4th day and to 10 per cent on the 5th and 7th days. After an exposure of 8 days the virus failed to kill the embryos. See Table VII.

The quantitative infectivity tests reflected to a marked degree the increased inactivation of the virus at 37°C as compared to room temperature and to 4°C. After one day of exposure the titer decreased from $10^{-9.32}$ to $10^{-5.5}$. The titer decreased from $10^{-5.5}$ to $10^{-3.62}$ on the 2nd day and to 10^{-3} on the 3rd day. The rate of inactivation was more pronounced between the 3rd and the 4th day than between the 1st and 2nd day. The titer decreased from 10^{-3} at the 3rd day to $10^{-0.21}$ at the 4th day. At the 5th day the virus was almost completely inactivated. See Table VII.

The decrease in hemagglutination titer of the virus did not follow the same pattern. Its thermostability was quite marked at

37°C. The hemagglutination titer decreased from + at 1280 to ± at 1280 on the 1st day and remained constant through the 7th day. The second decrease occurred at the 8th day from ± at 1280 to + at 640 and remained constant at the 9th day. See Table VIII.

Qualitative infectivity tests of the virus at 56°C showed that it was moderately resistant to this temperature. After 25 minutes exposure, the virus was still 100 per cent lethal to inoculated embryos. At 30 minutes and at 35 minutes the mortality rates were 70 per cent and 30 per cent, respectively. The virus was completely inactivated after 40 minutes exposure. See Table IX.

In the quantitative infectivity tests the titer of the virus decreased from $10^{-9.32}$ to less than 10^0 at 35 minutes exposure and the virus was completely inactivated at 40 minutes exposure. See Table IX.

The thermostability of the hemagglutinating activity of the virus was quite marked. It was only slightly impaired after 5 minutes during which period the titer decreased from + at 1280 to ± at 1280. The gradual progressive decrease in titer continued and at 90 minutes the titer was + at 40. See Table X.

The qualitative infectivity tests of the virus exposed to 62°C showed a rapid inactivation of the virus. All the embryos were killed by the virus subjected to this temperature for 5 minutes. The mortality rate was 70 per cent after 10 minutes and complete inactivation occurred in 15 minutes. See Table XI.

Quantitative infectivity tests showed a rapid decrease of titer from $10^{-9.32}$ to about 10^{-1} at 5 minutes and to $10^{-0.38}$ at 10 minutes. Virus heated for 15 minutes was completely inactivated. See Table XI.

The hemagglutinating activity was more thermostable than the infectivity. The hemagglutination titer decreased from + at 1280 to + at 640 in 5 minutes, + at 320 at 10 minutes, + at 80 at 15 minutes and ± at 20 at 20 minutes. Virus heated for 25 minutes failed to agglutinate the red cells. See Table XII.

From comparisons of the data of heat resistance of the infectivity of the virus strain used in these experiments and of the hemagglutinating activity, it is apparent that the infectivity of the virus for embryos and its hemagglutinating property are inactivated independently. The presence of inactivated virus which could not be shown by embryo inoculation could be detected by hemagglutination tests.

The infectivity of the virus for chicken embryos was more thermolabile at 4°C, room temperature (22°C- 27°C), 37°C, 56°C and 62°C than the hemagglutinating activity at these temperatures. Both activities were simultaneously destroyed at 77°C and 100°C during a one minute exposure period.

Studies of the data for 56°C would indicate that this strain of the virus resembles closely the McV strain studied by Hanson, Upton, Brandly and Winslow[22].

When unheated Newcastle disease virus is used in the hemagglutination test the virus is adsorbed on the chicken red blood cells causing the cells to agglutinate. After incubation for about an hour at room temperature the virus elutes from the cells which then settle to the bottom of the tube. With heat-treated Newcastle disease virus the elution may be delayed to a variable extent. Slight elution occurred after one hour and remained constant for an additional several hours with virus which had been heated at 56°C for 90 minutes. No elution occurred after two hours with virus which had been held at room temperature (22°C - 27°C) for 28 and 30 days.

The results would indicate that the reaction between heated virus and chicken red blood cells is firmer than with unheated virus. It is believed that the elution of the virus occurs as a result of an enzymatic destruction of the red cell receptors by active virus. The lack of elution is thought to be due to the inability of the heated virus to produce enzymatic destruction of the corresponding red cell receptors. From the present studies it would seem that the receptor destroying factor is slightly inactivated at 56°C for 80 and 90 minutes whereas the infectivity of the virus was inactivated after 40 minutes of exposure. This would indicate that the receptor destroying factor for this particular strain of the virus is more thermostable than the infectivity as tested with embryonating chicken eggs and it is more thermolabile than the hemagglutinating property of the virus.

TABLE I

Procedure for hemagglutination test

Virus dilutions

	Und.	$\frac{1}{5}$	$\frac{1}{10}$	$\frac{1}{20}$	$\frac{1}{40}$	$\frac{1}{80}$	$\frac{1}{160}$	$\frac{1}{320}$	$\frac{1}{640}$	$\frac{1}{1280}$	$\frac{1}{2560}$	$\frac{1}{5120}$	Control
Virus dilution ml.	0.25 ml. per tube												0
Saline ml. 0.85%	0.25 ml. per tube												0.5 ml.
Chicken red Blood cells 0.5% suspension	0.25 ml. per tube												0.25 ml.

TABLE II

Infectivity titration of Newcastle disease virus strain (48-9-328) *

Log of virus dilutions

10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	Titer	Infective doses in 0.1 ml. inoculum.
						4**	4**	5	3	1	0	$10^{-9.32}$	2,138,000,000

* 5 eggs inoculated per dilution
Figures in table represent the number of embryos that died.
** One egg in each dilution died within the first 24 hours post-inoculum.
These eggs were not included in the total number for the respective dilutions.
(This footnote applies also to Tables III, V, VII, IX, XI.)

Hemagglutination test of Newcastle disease virus strain (48-9-328)

Virus dilutions

1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	Control
+	+	+	+	+	+	+	+	+	-	-	-

+ Complete hemagglutination.
± Partial hemagglutination.
\- No hemagglutination.

(This footnote applies also to Tables IV, VI, VIII, X, XII.)

TABLE III

Infectivity titrations of virus at 4°C *

Log of virus dilutions

	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	Titer	Infective doses
3. day	9**													
1. week	10				5	5	5	5	4	3	1**	0	$10^{-9.17}$	1,479,000,000
9. day	10													
11. day	10													
2. week	10				5	5	5	5	5	2	0	0	$10^{-8.83}$	676,100,000
16. day	10													
3. week	10				5	5	5	5	5	0	0	0	$10^{-8.50}$	316,200,000
23. day	10													
4. week				5	5	5	5	5	5	2	1**		$10^{-9.00}$	1,000,000,000
6. week						5	5	5	3	0	1		$10^{-8.31}$	204,200,000
9. week				5	5	5	5	4**	4	-			$10^{-8.37}$	234,400,000
11. week						5	5	5	2	0	0		$10^{-7.83}$	67,610,000
13. week					5	5	5	5	1	0			$10^{-7.62}$	41,690,000

TABLE IV

Hemagglutination tests of virus at 4°C

Virus dilutions

	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	Control
1. week	+	+	+	+	+	+	+	+	+	-	-	-
2. week	+	+	+	+	+	+	+	+	+	-	-	-
3. week	+	+	+	+	+	+	+	+	+	-	-	-
4. week	+	+	+	+	+	+	+	+	+	-	-	-
6. week	+	+	+	+	+	+	+	+	+	-	-	-
9. week	+	+	+	+	+	+	+	+	+	-	-	-
11. week	+	+	+	+	+	+	+	+	+	-	-	-
13. week	+	+	+	+	+	+	+	+	+	-	-	-

TABLE V

Infectivity titrations of virus at room temperature (22°C - 27°C) *

	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	Titer	Infective doses
1. day	10		5	5	5	5	5	5	2	0	0	$10^{-7.83}$	67,610,000
3. day	10	5	5	5	5	5	5	5	2	0		$10^{-7.83}$	67,610,000
5. day	10	5	5	5	5	5	4	0	0			$10^{-6.37}$	2,344,000
7. day	10	5	5	5	5	5	5	2	0			$10^{-6.83}$	6,761,000
9. day	10	5	5	5	5	4	2	1	0			$10^{-5.84}$	691,800
11. day	10	5	5	5	5	1	0	0	0			$10^{-4.62}$	41,690
12. day	10	5	5	5	5	1	0	0	0			$10^{-4.62}$	41,690
13. day	10	5	5	5	3	0	0	0	0			$10^{-4.17}$	14,790
14. day	10	5	5	5	5	1	0	0	0			$10^{-4.62}$	41,690
15. day		5	5	5	1	0						$10^{-3.62}$	4,169
17. day		5	5	5	2							$10^{-3.83}$	6,761
19. day		5	5	1	1							$10^{-2.75}$	562
21. day		5	5	0	0							$10^{-2.50}$	316
22. day	9	5	2	0								$10^{-1.83}$	68
23. day	9	5	2	0								$10^{-1.83}$	68
24. day	8	5	0	0								$10^{-1.50}$	32
25. day	8	2	0									$10^{-0.61}$	4
27. day	7	2	0									$10^{-0.50}$	3
28. day	5	1	0									$10^{-0.10}$	1
30. day	3	0	0									$<10^{0}$	<1

TABLE VI

Hemagglutination tests of virus at room temperature (22°C-27°C)

Virus dilutions

	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	Control
1. day	+	+	+	+	+	+	+	+	±	-	-	-
3. day	+	+	+	+	+	+	+	+	±	-	-	-
5. day	+	+	+	+	+	+	+	+	±	-	-	-
7. day	+	+	+	+	+	+	+	+	±	-	-	-
9. day	+	+	+	+	+	+	+	+	±	-	-	-
11. day	+	+	+	+	+	+	+	+	±	-	-	-
12. day	+	+	+	+	+	+	+	+	±	-	-	-
13. day	+	+	+	+	+	+	+	+	±	-	-	-
14. day	+	+	+	+	+	+	+	+	±	-	-	-
15. day	+	+	+	+	+	+	+	+	±	-	-	-
17. day	+	+	+	+	+	+	+	+	±	-	-	-
19. day	+	+	+	+	+	+	+	+	±	-	-	-
21. day	+	+	+	+	+	+	+	+	-	-	-	-
22. day	+	+	+	+	+	+	+	+	-	-	-	-
23. day	+	+	+	+	+	+	+	+	-	-	-	-
24. day	+	+	+	+	+	+	+	+	-	-	-	-
25. day	+	+	+	+	+	+	+	+	-	-	-	-
27. day	+	+	+	+	+	+	+	+	-	-	-	-
28. day	+	+	+	+	+	+	+	+	-	-	-	-
30. day	+	+	+	+	+	+	+	+	-	-	-	-

TABLE VII

Infectivity titrations of virus at 37°C *

	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	Titer	Infective doses
1. day	10					5	0	0	0	0	0	0	$10^{-5.5}$	316,200
2. day		5	5	5	1	0							$10^{-3.62}$	4,169
3. day	10			0	0	0	0	0	0	0	0		$<10^{-3}$	3,000
4. day	5	1	1	0									$10^{-0.21}$	6
5. day	1		0	0	0	0	0	0	0	0	0		$<10^0$	<1
7. day	1	0	0	0	0	0							$<10^0$	<1
8. day	0	0	0	0	0	0								
9. day	0	0	0	0	0	0								

TABLE VIII

Hemagglutination tests of virus at 37°C

Virus dilutions

	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	Control
1. day	+	+	+	+	+	+	+	+	±	-	-	-
2. day	+	+	+	+	+	+	+	+	±	-	-	-
3. day	+	+	+	+	+	+	+	+	±	-	-	-
4. day	+	+	+	+	+	+	+	+	±	-	-	-
5. day	+	+	+	+	+	+	+	+	±	-	-	-
7. day	+	+	+	+	+	+	+	+	±	-	-	-
8. day	+	+	+	+	+	+	+	+	-	-	-	-
9. day	+	+	+	+	+	+	+	+	-	-	-	-

TABLE IX

Infectivity titrations of virus at 56°C *

Log of virus dilutions

	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	Titer	Infective doses
5 minutes	10	5	5	5	5	5	5	5		$\geqq 10^{-7}$	$\geqq$ 10,000,000
10 minutes	10	5	5	5	5	5	2	0		$10^{-5.83}$	676,100
15 minutes	10	5	5	4	2	1	0			$10^{-3.84}$	6,918
20 minutes	9**	5	5	2	0					$10^{-2.83}$	676
25 minutes	10	4	1	1						$10^{-1.62}$	42
30 minutes	7	3	1	0						$10^{-1.32}$	21
35 minutes	3	1	0							$< 10^{0}$	< 1
40 minutes	0										

TABLE X

Hemagglutination tests of virus at 56°C

Virus dilutions

	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	Control
5 minutes	+	+	+	+	+	+	+	+	±	-	-	-
10 minutes	+	+	+	+	+	+	+	+	-	-	-	-
15 minutes	+	+	+	+	+	+	+	+	-	-	-	-
20 minutes	+	+	+	+	+	+	+	±	-	-	-	-
25 minutes	+	+	+	+	+	+	+	±	-	-	-	-
30 minutes	+	+	+	+	+	+	+	-	-	-	-	-
35 minutes	+	+	+	+	+	+	+	-	-	-	-	-
40 minutes	+	+	+	+	+	+	±	-	-	-	-	-
45 minutes	+	+	+	+	+	+	±	-	-	-	-	-
50 minutes	+	+	+	+	+	+	±	-	-	-	-	-
55 minutes	+	+	+	+	+	+	-	-	-	-	-	-
60 minutes	+	+	+	+	+	+	-	-	-	-	-	-
70 minutes	+	+	+	+	+	±	-	-	-	-	-	-
80 minutes	+	+	+	+	+	-	-	-	-	-	-	-
90 minutes	+	+	+	+	-	-	-	-	-	-	-	-

TABLE XI

Infectivity titrations of virus at 62°C. *

	Log of virus dilutions				
	10^{0}	10^{-1}	10^{-2}	Titer	Infective doses
5 minutes	10	5		$\geqq 10^{-1}$	$\geqq 10$
10 minutes	7	1	0	$10^{-0.38}$	2
15 minutes	0				

TABLE XII

Hemagglutination tests of virus at 62°C.

Virus dilutions

	Und.	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	Control
5 minutes	+	+	+	+	+	+	+	+	+	-	-	-	-
10 minutes	+	+	+	+	+	+	+	+	-	-	-	-	-
15 minutes	+	+	+	+	+	+	-	-	-	-	-	-	-
20 minutes	+	+	+	±	-	-	-	-	-	-	-	-	-
25 minutes	-	-	-	-	-	-	-	-	-	-	-	-	-
30 minutes	-	-	-	-	-	-	-	-	-	-	-	-	-
35 minutes	-	-	-	-	-	-	-	-	-	-	-	-	-
40 minutes	-	-	-	-	-	-	-	-	-	-	-	-	-
45 minutes	-	-	-	-	-	-	-	-	-	-	-	-	-

SUMMARY

The results obtained in the present study show that the hemagglutinative activity of the strain of Newcastle disease virus used was more thermostable than the infectivity of the virus.

The initial LD_{50} of the virus strain was $10^{-9.32}$ and its hemagglutination titer was + at 1280.

At 4°C the infectivity titer of the virus decreased to $10^{-7.62}$ in 13 weeks whereas the hemagglutination titer remained constant for this period.

At room temperature (22°C - 27°C) the infectivity of the virus decreased to $< 10^{0}$ in 30 days. The hemagglutination titer decreased to + at 640.

At 37°C the infectivity titer decreased to $< 10^{0}$ in 7 days and was negative in 8 days and the hemagglutination titer decreased to 640 in 9 days.

At 56°C the infectivity of the virus decreased to $< 10^{0}$ in 35 minutes and was negative in 40 minutes. The hemagglutination titer decreased to 40 in 90 minutes.

At 62°C the infectivity titer decreased to $10^{-0.38}$ in 10 minutes and was negative in 15 minutes. The hemagglutination titer decreased to 10 in 20 minutes and negative in 25 minutes.

At 77°C and 100°C the infectivity titer decreased to negative within one minute and the hemagglutination titer to negative within the same time interval.

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