HEMAGGLUTINATION-INHIBITION ANTIBODIES IN EGG-YOLK FROM HENS VACCINATED WITH "LEUCOSIS-CONTAMINATED" AND "LEUCOSIS-FREE" NEWCASTLE DISEASE VACCINE

By

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Object:

The purpose of this experiment was to determine if there were any differences in the hemagglutination-inhibition antibody titers present in yolk from eggs laid by hens vaccinated with live-virus Newcastle disease vaccines prepared from "leucosis-contaminated" and from "leucosis-free" embryonating chicken eggs.

This experiment was made possible through a cooperative project of the Michigan Agricultural Experiment Station and the United States Department of Agriculture, Regional Poultry Research Laboratory, East Lansing, Michigan, supported in part by a grant-in-aid from the National Cancer Institute, in the possible transmission of the agent of avian leucosis through embryonating chicken eggs used in the production of avian virus vaccines. The experiment was designed as one phase of the project since the eggs were available and afforded an unusual opportunity to study the effect of the various vaccines on the hemagglutination-inhibition antibodies.

REVIEW OF LITERATURE

Newcastle Disease Virus

What is now generally considered as Newcastle disease was first reported by Kraneveld[1] in 1926 as a highly diffusible and fatal infection of poultry prevalent in the Dutch East Indies.

Doyle[2] in 1927 originally isolated the virus of Newcastle disease during an epizootic amongst fowls near Newcastle-on-Tyne, England. Doyle was successful in demonstrating that the causative agent was a filterable virus. He reported that infected lung, liver, spleen and brain which were dried in a dessicator over $CaCl_2$ in the cold were active at 40 and 100 days, but the ovary was negative at both time intervals. Virus in these various organs in a 50 per cent solution of glycerine in physiologic saline was active after 197 days, but was dead after 259 days. In physiologic saline, the virus was active up to 86 days, but inactive at the hundredth day. Virus in a spleen kept in a Petri dish was active after 80 days, but was inactive after 150 days. Blood in sealed pipettes was active after 109 days. In the only experiments on the effects of temperature on the virus, the virus was active after 48 hours at 37° C. and inactive after 72 hours. Cross immunity tests proved that the virus of Newcastle disease and fowl plague were

separate entities.

Burnett and Ferry[3] indicated by comparative filtration studies with virus-infected allantoic fluid that Newcastle disease virus was from 80 to 120 mu and fowl plague virus was 60 to 90 mu. They also showed that Newcastle disease virus was more resistant to photodynamic inactivation by methylene blue than was fowl plague virus. These differences in conjunction with known clinical and immunological differences pointed to a complete etiological independence of the two diseases.

Stover[4] identified a filterable virus as the causative agent of a "nervous disorder" and "respiratory nervous disorder" of chickens in California in 1942. The "respiratory nervous disorder" described by Stover was named "avian pneumoencephalitis" by Beach.[5] He stated that the disease had been present in California in 1935 or earlier. In 1944 Beach[6] showed that "avian pneumoencephalitis" was immunologically related to Newcastle disease virus. The clinical picture of the disease caused by the respective agents differed somewhat. In this country Newcastle disease is associated with a comparatively low mortality rate in adult chickens and with a high mortality rate in chicks. In other countries, the disease is accompanied by a 100 per cent mortality rate.

According to Beaudette[7] Newcastle disease virus may be found in the following material in the avian species: saliva, feces, liver, spleen, lung, pancreas, bone marrow, kidney, egg yolk, testes, intestines and contents, bile, edematous material, crop contents, brain and cord, blood and pericardial fluid.

Burnett[8] reported that outbreaks of Newcastle disease have been described chiefly from Asiatic countries. The disease seems to be endemic in Japan, the Indies and the Phillipine Islands.

Chu et al[9] reported that epizootic diseases of fowls resembling Newcastle disease have been reported in various parts of China and that Newcastle virus was isolated and identified. They reported some minor clinical differences in the Chungking strain and the Nanking strain, and between the United States strains and those isolated in other parts of the world.

In 1948, Osteen and Anderson[10] reported that Newcastle disease had been diagnosed in 42 states and the District of Columbia.

Cunha et al[11] reported that Newcastle disease virus was a complex of which approximately 67 per cent was protein and approximately 27 per cent was lipid material. A relatively small amount of nucleic acid, some of which was of the desoxypentose type was also present. They reported that the infectivity of the

purified material was such that $10^{-13.72}$ grams of Newcastle disease virus constituted the 50 per cent end point unit of infectivity for chick embryos.

Howitt et al[12] found neutralizing antibodies of Newcastle disease in the blood of various groups of children and in some adults suffering from a mild central nervous system illness with a short, non-fatal course. These authors suggested the probability that poliomyelitis-like, mild, central nervous system infections, especially in children, and influenza-like diseases in adults may be contracted from chicks affected with Newcastle disease.

Immunity conferred passively via the egg in birds, in utero or via the colostrum in mammals, has long interested immunologists.[13]

During the Italian epizootics, Vianello[14] found that chicks which were the progeny of recovered hens resisted Newcastle disease, whereas those hatched from eggs from other sources died en masse.

Brandly et al[15] revealed the transference of Newcastle disease antibodies from immune hens to egg yolk and the developing embryo and extra-embryonic tissues. Significant quantities of specific antibodies were present in extracts of embryo tissue and of the allantoic and amnionic membranes after 6 to 9 or more days of incubation. Antibodies were not detected in the serum of

embryos until the 15th day of incubation. Demonstration of antibodies in blood serum paralleled maturation of the developing embryo.

Rywosch[16] and Polk et al[17] showed that the presence of antibodies was not due to the development of complement or specific amboceptor in embryonating chicken eggs. These authors were unable to demonstrate complement in embryo serum until the 21st day. Antibodies could not be demonstrated until the 17th day in the young chick and then only in a low concentration.

Brandly et al[15] reported that complement was unnecessary and exerted no appreciable effect or specific neutralization of Newcastle disease virus by serum. An antibody titer could be demonstrated for the first two weeks of postnatal life. This was followed by a rather rapid decline in serum antibodies and immunity. It was shown that the antibody titer decreased with absorption of yolk. Congenital passive immunity conferred a high degree of refractivity to Newcastle disease virus. Congenital or passive immunity interfered with the active immunity response of young chicks to vaccination. An egg-yolk antibody titer of 10^2 or more neutralizing doses would suggest a recent association of hens with Newcastle disease virus. According to these authors, egg yolk may be used instead of blood serum for assessment of previous Newcastle infection.

Schmittle and Millen[18] described a technique whereby they were able to detect the presence of antibodies in unincubated eggs by means of the hemagglutination-inhibition test. Egg yolk previously treated with ethylene dichloride and ethyl ether was used in place of serum in the test.

Prier et al[19] reported the presence of active Newcastle virus in the yolks of 2 out of 50 eggs from 18 experimental vaccinated birds, 4 and 5 days, respectively, after vaccination. Virus was not found in eggs at various periods of 1 to 26 days after challenge exposure of the vaccinated hens. Live virus was found in one egg from a hen naturally affected with Newcastle disease before she was experimentally inoculated with live Newcastle virus vaccine. No virus was found in eggs from hens that had resumed normal egg production following vaccination.

Newcastle antibodies were found in eggs as early as 19 days after vaccination, and were still present in one group of eggs 109 days after vaccination.

De Lay[20] reported the isolation of Newcastle virus by chick embryo culture from the yolk of 6 four-day-old chicks and from embryos of the succeeding hatch dead on the 15th day of incubation.

Avian Leucosis Complex

Caparini in 1896, according to Olson,[21] was the first to record leucosis in chickens.

Jungherr[22] reported that the term leucosis signifies a group of diseases characterized by autonomous proliferation of the precursors of leucocytes. It was considered that the avian leucosis complex is comprised of the following: fowl leucosis, granulobastosis, lymphomatosis, lymphoid tumors and neural lymphomatosis. Vitamin E deficiency also caused a "lymphoblastoma"-like condition in chicks. The genetic aspects in the transmission of leucosis are highly complex. They are intimately associated with the unsettled questions of etiologic classification and gonadal transmission of the avian leucosis complex. The filterable agent of visceral lymphomatosis affects primarily young birds. Immunological methods to differentiate interrelationships among transmissible agents of the avian leucosis complex are frequently impeded by resistant birds and by a lack of immunizing properties of the agents.

Ellermann et al[23] conducted extensive experimental studies on the conditions of leucosis in the common fowl. They recognized three general forms of avian leucosis, namely, leucemic or aleucemic myeloid leucosis, intravascular "lymphoid", i.e., erythroid

leucosis, and extravascular lymphatic leucosis, all of which were considered to be transmissible and caused by the same filterable virus.

Emmel[24] considered the following non-specific factors, which may initiate blood changes, to be basic in the development of avian leucosis complex: iron, Salmonella toxins, vitamin A and K deficiencies and poor ventilation.

Winton et al[25] demonstrated that visceral lymphomatosis might be induced by inoculation with a filterable agent which is found in the tumor tissue, and in the blood of chickens with the disease. The presence of the agent in certain chick embryos and in day-old chicks was also demonstrated.

According to Jungherr,[22] the concept that the cause of avian leucosis complex is unknown, is essentially incorrect, because etiologic agents can be identified by methods peculiar to virology. Jungherr was of the opinion that eggs could be carriers of the avian leucosis complex. He also proposed the possibility that fowl pox vaccine produced from leucosis-contaminated embryonating chicken eggs could be capable of transmitting the avian leucosis complex when such vaccines were used for immunization.

Doyle[26] was the first to suggest that avian lymphomatosis (fowl paralysis) could be transmitted

through the egg.

Van der Walle and Winkler-Junius in Holland in 1924[27] reported the first positive transmission of fowl paralysis.

Cottral et al[28] reported the transmission of lymphomatosis from parents to offspring through hatching eggs by injecting embryonic and chick tissues into susceptible chickens. Only chicks and embryos that had no gross abnormalities and which were from offspring of "normal" sires and dams that had lived for 600 days free of all clinical or gross evidences of lymphomatosis were used. Chicks were inoculated with suspensions of pooled liver tissue from 15-day embryos, 18-day embryos, newly hatched chicks and with filtrates of liver tissue from newly hatched chicks. After 300 days, the respective groups of chickens that were inoculated had an incidence of lymphomatosis as follows: 82.4 per cent, 100.0 per cent, 76.5 per cent and 85.7 per cent as compared to an incidence of 17.6 per cent in uninoculated controls. These workers reported that only visceral lymphomatosis was transmitted by these means.

Kissling[29] reported positive results for leucoagglutination as a serological diagnosis for avian lymphomatosis. Of 94 samples of chicken sera, 48 showed the presence of lymphocyte agglutinins. Of the 48

samples, 40 (83.3 per cent) showed lesions of the leucosis complex.

Winton et al[25] have not obtained consistent results with the following diagnostic tests for the detection of lymphomatosis: (1) the hemagglutination of chicken red cells with lipid antigens, (2) the Kahn test, (3) heat coagulation of plasma and (4) the indirect complement fixation test.

Interference Phenomenon

Hoskins reported[30] that the presence of a certain strain of a virus in a susceptible tissue sometimes prevented the growth of a related strain in the same tissue; for example, inoculation of a monkey with both virulent-pantropic and avirulent neurotropic strains of yellow fever virus did not result in infection, provided the neurotropic strain was injected within 20 hours after the pantropic strain was administered. He also demonstrated that interference could occur between agents, so far as was known, that were antigenically unrelated.

Findlay and MacCallum[31] studied the interference between the virus of Rift Valley fever and the two strains of yellow fever. Rift Valley fever was serologically unrelated to yellow fever and no cross immunity was induced. The neurotropic yellow fever virus protected mice against Rift Valley fever virus; and the Rift Valley fever virus protected monkeys against pantropic

yellow fever virus. The authors proposed that manifestation of interference possibly resulted from the inability of virus particles to invade cells which were occupied by other actively multiplying virus particles.

Andrewes in 1942[32] demonstrated interference between strains of influenza A virus in tissues. Inoculation of 9 mice with 0.02 cc. of vaccine prepared from W. S. strain of influenza A followed by inoculation with 0.02 cc. of a preparation of vaccine from a neurotropic variant of the same virus strain resulted in the death of only one mouse.

Sharples et al[33] reported that the following neurotropic viruses possessed oncolytic powers: Russian Spring Summer virus, West Nile virus, Japanese B encephalitis virus, St. Louis encephalitis virus and louping ill virus. It was demonstrated that chickens inoculated with tumor and with any of the above viruses were usually rendered immune to further tumor challenge even though the original inoculum did not produce a palpable tumor.

Ziegler and Horsefall[34] demonstrated interference between strains of the two serologic types of influenza A in chick embryos. These workers found that embryos inoculated with either type of virus were refractory to infection to the other type within 8 to 12 hours after inoculation. Likewise, the introduction of

a very large amount of one type caused interference with the multiplication of the other type when injected simultaneously. The interference between influenza B strains did not render embryos insusceptible to infection from a large inoculum of influenza A strains.

Henle and Henle,[35] reported that the reciprocal interference between strains of influenza A and B viruses in the chick embryo had been as readily demonstrable with noninfective preparations as with active viruses; although when a noninfective virus was employed, large inocula were necessary to cause interference. A few hours after the establishment of infection in the chick embryo, additional multiplication of virus was inhibited by the injection of a large amount of noninfective virus. Preparations which showed interference caused by noninfectious virus particles, "auto-interference", could be produced by prolonged incubation of the inoculated chick embryo, prolonged storage of infected allantoic fluid at 4° C., heating and ultraviolet irradiation.

Ziegler *et al*[36] reported that the capacity to produce interference was inactivated by ultraviolet irradiation or by heat at a less rapid rate than was the property of infectiousness. Interference apparently was caused by the altered virus particle itself. The property could not be separated from the virus and was

specifically neutralized by the homologous type antiserum.

They also reported the well-established facts that strains might not be successfully propagated if serial passage was carried out with undiluted allantoic fluid, and that higher virus titers were obtained when dilute inocula were employed, might be attributed to "auto-interference". It was the opinion of this group that interference had been dependent upon the capacity of virus particles to react with susceptible cells, and that when quantitative saturation of such cells by virus particles had been achieved, it was not possible to establish infection with the same or other virus strains, irrespective of their serologic type.

Henle and Henle[37] stated that interference was caused with great speed by the following: pathological changes in the cell, exhaustion of metabolites that are essential for an enzyme or amino acid production, and production of antibodies.

Hirst[38] reported that interference was mediated by a specific cell receptor. Such a receptor could have been reactive with both infective or noninfective virus particles if the inactive virus particle retained the particular or specific structure of arrangement required for reception. Hirst concluded that interference could have been quantitative

saturation of receptors by three means:

1. Multiplication of active virus (auto-interference)
2. Introduction of large amounts of active virus against infectious virus present, a competition for receptors
3. Saturation with noninfectious ultraviolet inactivated virus particles.

Rivers[39] indicated that genetic experiments on the specific virus must be taken into account in seeking an explanation for the interference phenomenon. He also reported that closely related virus particles growing together freely in a single cell were indicative of the idea of competitive growth. Since the initial rate of growth of a particular virus in the cell was likely to be proportional to the amount of that virus present, competition for the substrate was sufficient to account for the observation that the virus which happened to get started first might determine which member of the pair would grow.

Rivers postulated that the successful virus swamped the synthetic mechanisms of the cell and under these conditions, the less favored virus, after having made an abortive attempt to grow, died. Evidence that this virus would die had been derived from material compiled on genetic exchange. If there should be an exchange of parts of two unrelated viruses, most of the

hybrid products would not be expected to be viable. Therefore, only the virus present in numerical excess would survive. This idea was in accord with the mutual exclusion and depressor effects of mixed infection.

Hemagglutination Activity

Hirst,[40] in 1941, was the first to notice that when allantoic fluid from chicken embryos previously infected with influenza A virus was being removed, the red cells coming from the ruptured vessels agglutinated in the allantoic fluid. It was also demonstrated that influenza B and swine influenza viruses were able to agglutinate red cells as well as influenza A. It was shown that when infectious allantoic fluid, fresh or stored at —72° C., from which the embryonic red cells had been removed by low-speed centrifugation, agglutinated normal adult chicken red cells. The agglutinated cells formed a ragged, granular pattern at the bottom of a test tube. The pattern produced by a mixture of noninfectious allantoic fluid and red cells was a sharp, round disc of cells. Hirst[41] later showed that the addition of specific immune serum inhibited hemagglutination of red cells in the presence of the homologous virus, but not when heterologous strains were used.

Hirst[41] prepared infectious allantoic fluid for his studies by inoculating 11-day-old white Leghorn embryos by the allantoic route with four different strains

of influenza virus: PR8 and W. S. strains of influenza A virus, the Lee strain of influenza B virus and swine influenza virus. A 2.0 per cent saline suspension of adult chicken red cells was used in this test. Titration of the hemagglutinating ability of the viruses was determined from serial 2-fold dilutions of the viruses in physiologic saline. Equal amounts of each virus dilution and red cell suspension were combined. These mixtures were immediately shaken and incubated at room temperature for one hour.

Serum to be tested for the presence of hemagglutination-inhibition substances was diluted in 2-fold steps in saline. To 0.5 ml. of each serum dilution was added 0.5 ml. of influenza virus-infected allantoic fluid diluted from 10^{-3} to 10^{-5} with physiologic saline. The tests were incubated for one hour at room temperature.

In both the virus and the serum inhibition titrations, readings were made by comparing the density of the suspensions with the density of known suspensions of red cells in saline. Hirst used the amount of red cell sedimentation as indicated by the clearing of the supernate as the index of the degree of hemagglutination rather than the pattern of agglutinated cells in the bottom of a tube.

Standard suspensions of red cells for comparison were prepared in concentrations of 1.0, 0.75, 0.50 and 0.30 per cent. The density of virus and red cell suspensions which were between the 1.0 and 0.75 per cent density

of the standard were designated as +, 0.75 and 0.50 per cent ++, 0.50 and 0.30 per cent +++, and less than the 0.30 per cent ++++.

In contrast with the small, sharply-outlined disc in the bottom of the negative control, the size of the disc in the bottom of the tube increased with increasing degrees of hemagglutination. The margins of the discs frequently had a characteristic irregular, lacy pattern composed of clumps of cells in those tubes in which hemagglutination had occurred. This type of pattern was prevalent with only slight degrees of hemagglutination, and there was no decrease in the density of the supernate. This type of reaction was designated ±.

The end-point of the titration of the hemagglutinative ability of the virus or of the hemagglutination-inhibition ability of the serum was arbitrarily taken as the dilution where ++ agglutination occurred. If ++ agglutination did not occur in any tube, the end-point was assumed to be half way between two serial dilutions, one showing more and the other less than ++ agglutination.

Temperature differences between 27° to 37° C. resulted in no obvious differences in the end-point in the agglutination test.

Hirst demonstrated that when almost all the virus was active, the infectivity of the virus could be destroyed without destroying the capacity to agglutinate red blood cells. Infectivity was destroyed at 56° C. for 15 minutes and at room temperature for several days. The fact that eggs inoculated with a number of different influenza virus strains yielded allantoic fluids with approximately the same hemagglutination titer suggested that the virus particle concentrations were approximately the same and that these different virus strains had similar hemagglutinative activity. He concluded that the hemagglutination test provided a simple way to determine the relative concentration of virus particles independent of virulence.

Hirst and Pickles[42] described a photo-electric densitometer which made possible a greater accuracy in determining the degree of clearing of the red cell suspensions.

Hirst[43] proposed that the mechanism of agglutination of chicken red cells by suspensions of influenza virus was similar to the adsorption of bacteriophage onto susceptible, specific receptors of bacterial cells. After having attached themselves, the "phage" particles multiplied, but there was no evidence that the influenza virus multiplied on the chicken red cells. After a

suitable incubation period, the virus particles were eluted from the red cells.

Hirst[44] reported that the variations in serum titers following influenza infection were due, to a considerable measure, to the use of red blood cells from different chickens. He noted that there were variations in the serum inhibition with different preparations of the same virus strains. He also showed that the agglutination method was sensitive enough to measure small, strain differences, and his findings correlated with results of neutralization tests.

Miller and Stanley[45] made a study of several variables encountered in chicken red blood cell agglutination tests for the presence of influenza virus. The best results were obtained when the ingredients had been incubated at 24° C. The hydrogen ion activity had a negligible effect on the titer between the range of pH 6.0 to pH 8.0. Variation of results could also be due to the specific behavior of the red blood cells from different chickens and to an instability of the red cells.

Salk[46] reported that temperature had a marked effect on the hemagglutination titer of influenza virus. At 20° C., higher titers resulted than at 35° C. due to the rapid rate at which the virus became dissociated from the cells at the higher temperature. At 22° C. and 25° C. identical titers were obtained which were between the

titers at 2° C. and 35° C.

Salk described a simple method for titrating the hemagglutinating capacity of influenza virus and the corresponding antibody. A suspension of 0.25 per cent red cells made from a 10.0 per cent stock suspension which had been stored at 4° C. were used. Salk reported that if the cells were kept under sterile conditions, they could be used for more than a week. With complete hemagglutination (+), the clumps of agglutinated red cells adhered to the bottom of the tube at the point of settling and were characterized by a dispersed rather than an aggregated pattern. A negative pattern (0) consisted of a central, sharply outlined disc. Intermediate reactions (±) appeared as irregular clumps of cells usually associated with a halo of finely aggregated or unagglutinated cells. The highest dilution of the virus which produced the maximum agglutination 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.

Brandly *et al*[47] reported that from a serological viewpoint, there were two procedures for hemagglutination-inhibition tests for Newcastle disease: the "alpha" and the "beta" methods. In the "alpha" method, successive dilutions of virus were mixed with a fixed amount of serum. In the "beta" method, successive

dilutions of serum were titrated against a predetermined amount of virus. This amount, 10 hemagglutinating units (HA units), was prepared from the greatest dilution of virus in which at least ++ hemagglutination occurred. This dilution was considered to contain one HA unit. If the end-point of the virus was 1:320, then 0.25 ml. of a 1:32 dilution of the virus was used as representing 10 HA units in the test. There was a 93 per cent agreement between the two methods which seemed sufficiently satisfactory to warrant the use of either one for diagnosis.

Storage of allanto-amniotic fluid virus in the unfrozen state for intervals of several months did not result in an appreciable loss of hemagglutination titer. Cells from different chickens varied in their reaction with Newcastle disease virus.

Burnet[8] reported that a Victorian strain of Newcastle disease virus was capable of agglutinating red blood cells from the following species: chicken, man, sheep I, guinea-pig, mouse, fowl, sparrow and frog.

Lush[48] showed that the hemagglutination test was applicable to Newcastle disease virus and to the fowl plague virus. Hemagglutination-inhibition tests showed that there was no serological relationship between these two viruses.

Beach[49] reported the application of the hemagglutination-inhibition Newcastle disease test in the diagnosis

of avian pneumoencephalitis.

Brandly et al[50] described a method for titrating the hemagglutinating ability of Newcastle disease virus and its homologous antibody. For the hemagglutination test, serial 2-fold dilutions of the virus were prepared in saline, and to 0.25 ml. of each virus dilution was added 0.25 ml. of physiologic saline. The contents of the tubes were thoroughly mixed and 0.25 ml. of a 0.5 per cent red cell suspension was added and tubes were shaken. The test was incubated at room temperature (23° to 27° C.). Readings were made at 15, 30 and 45 minute intervals. Hemagglutination was indicated as + (positive) and — (negative).

For the hemagglutination-inhibition test, 0.25 ml. amounts of 1:5 diluted positive, negative and suspect serums were added in place of the saline in the hemagglutination test, and the results were read exactly like the hemagglutination test. In this test, it was necessary to include the hemagglutination test in order to determine the titer of the virus.

The hemagglutination titer of the virus was the highest virus-dilution, before the addition of saline and cells, in which a positive pattern was present. The titer of the serum was the lowest virus-dilution, before the addition of serum and cells, in which a negative

pattern was present. To calculate the serum hemagglutination-inhibition titer, the titer of the virus was divided by the titer of the serum. Multiplication of the quotient by the dilution factor of the serum gave the serum hemagglutination-inhibition titer.

These authors reported that formalinized virus should be stored in the refrigerator. Virus which was more than 60 days old and which showed a decrease in hemagglutinating activity should not be used. In this test, turkey serum could not be used satisfactorily with chicken red cells because the serum of turkeys possessed a normal agglutinin for chicken cells.

Burnet et al[51] demonstrated that red blood cells which had been previously agglutinated by an excess of Newcastle disease virus were "sensitized" by the virus. "Sensitized" cells after washing twice with saline at room temperature could be agglutinated by influenza A and B, swine influenza, W. S. strain of influenza A and vaccinia, but not by Newcastle disease virus.

These authors reported that the inhibition effect of human tears on hemagglutination by influenza virus might be related to known non-specific inhibition by certain normal sera and to inhibition by a component of normal allantoic fluid studied by Rawlinson (unpublished) in Burnet's laboratory.

Anderson[52] showed that cells sensitized as

reported by Burnet[51] were able to agglutinate normal chicken red cells. Anderson's experiments indicated that agglutination by sensitized cells was due to the relatively firm adsorption of Newcastle disease virus particles on the surface of the cell. The cell sensitizing agent (CSA) which had been adsorbed on the surface of the cells, even though thoroughly washed, retained its infectivity for the chick embryo. Agglutinins on stable cells were neutralized by Newcastle disease immune sera, and the serum titers corresponded closely with the direct hemagglutination-inhibition titers.

Burnet and Anderson[53] indicated that the changed character of sensitized red blood cells was due to the adsorption to their surface of an agent other than the virus produced during growth of Newcastle virus in chick embryo cells.

They also reported that human red blood cells treated with amniotic or allantoic fluid preparations of Newcastle disease virus developed a new antigenic character which allowed them to be agglutinated to a high titer either by experimental Newcastle disease virus immune serum or by most sera from recent cases of infectious mononucleosis in man.

Osteen and Anderson in 1948[10] demonstrated that diluting Newcastle disease positive serum with 9 parts of negative serum did not lower the hemagglutination-

inhibition titer. Diluting with 19 parts of negative serum resulted in a 10-fold decrease in titer. These workers believed that serious error because of dilution was improbable. A hemagglutination-inhibition titer of 80 or more was considered to be diagnostic of prior Newcastle infection. Diagnosis of Newcastle disease by the hemagglutination-inhibition test was considered as accurate and as reliable as the serum neutralization test.

Hanson *et al*[54] reported the presence of normal hemagglutinins in the supernatant fluid of centrifuged 11- to 13-day yolk-sac membrane suspensions. The reaction was indistinguishable from serum hemagglutination-inhibition. They observed that the normal hemagglutinins in both normal and Newcastle disease infected yolk-sac membranes, completely "masked" the virus hemagglutinative activity and made the demonstration of the latter impossible. These workers demonstrated that normal chicken serum, added in the same manner and amount as the immune serum in the virus hemagglutination-inhibition test, inhibited the normal yolk-sac hemagglutination reaction and the virus hemagglutinin so that it could be demonstrated. Newcastle disease virus immune serum inhibited both normal and virus hemagglutinative activity.

Beach[49] proposed two reasons for serums which had the same virus-neutralizing titers, but which had a wide hemagglutination-inhibition titer range: hemagglutination-inhibiting and virus-neutralizing antibodies might be independent substances but an independent substance which suppressed the hemagglutination-inhibition reaction might be present in some serums. He also stated that the hemagglutination-inhibition test alone was considered inadequate to establish the presence of a Newcastle disease in an area previously believed free of Newcastle disease virus.

EXPERIMENTAL PROCEDURE

The presence of Newcastle disease antibodies in unincubated egg-yolk from hens vaccinated with "leucosis-contaminated" and "leucosis-free" Newcastle disease vaccine was evaluated by means of the hemagglutination-inhibition tests.

For a period of 10 consecutive weeks, beginning on February 3, 1950, 5 eggs were collected weekly at random from each of the 5 vaccinated groups of hens and from the control group. These chickens had been vaccinated from 19 to 25 weeks prior to the initial collection of the eggs.

Vaccines

All eggs used for the production of the experimental live-virus Newcastle disease vaccines and all chickens used for studies of the possible transmission of the agent of avian leucosis through embryonating chicken eggs used for the production of avian virus vaccine were supplied by the United States Department of Agriculture, Regional Poultry Research Laboratory, East Lansing, Michigan.

The vaccines were prepared in the Section of Virology, Department of Bacteriology and Public Health by Dr. Charles H. Cunningham who vaccinated the experimental chickens in cooperation with Dr. George E.

Cottral, Regional Poultry Research Laboratory.

Live-virus Newcastle disease vaccines were prepared from "leucosis-contaminated" and from "leucosis-free" eggs. These vaccines were designated as No. 707 and No. 1435-1436, respectively.

No. 707 vaccines were prepared in embryonating chicken eggs laid by hens which, through previous progeny tests, had been shown to be transmitting the virus of avian lymphomatosis through the ovarian route. The eggs were considered to be "leucosis-contaminated".

No. 1435-1436 vaccines were prepared in embryonating chicken eggs laid by hens of "leucosis-free" stock of the Regional Poultry Research Laboratory. These hens were from Inbred Line 15 which had shown a 0 to 20 per cent incidence of lymphomatosis over a period of 10 years.

Three types of vaccines were prepared from Nos. 707 and 1435-1436. The first type consisted of 1/2 of the liver from the Newcastle virus-infected embryo. The second type consisted of the remaining 1/2 of the liver and 1/2 of the extra-embryonic fluids and ground Newcastle disease virus-infected embryo. The third type consisted of Newcastle disease virus-infected ground extra-embryonic fluids, chorio-allantoic membrane and entire embryo.

All vaccines were applied by the "wing-web or stick method" to 30-day-old Single Comb White Leghorns, Inbred Line 15. Cottral *et al*[28] had previously

demonstrated by artificial infection that chickens from Line 15 were susceptible to lymphomatosis.

The vaccinated chickens were housed in a building containing 5 isolation pens.

In Pen I, 51 females were vaccinated with No. 707, Type 1 vaccine.

Pen II contained 50 females which were vaccinated with No. 707, Type 2 vaccine.

In Pen III, 25 chickens, the majority of which were males, were inoculated with No. 707, Type 2 vaccine. The remaining 25 males in Pen III were vaccinated with No. 707, Type 3 vaccine.

Pen IV contained 50 females which were vaccinated with No. 1435-1436, Type 3 vaccine.

Pen V contained 50 chickens, the majority of which were males, which had been vaccinated with No. 1435-1436, Type 3 vaccine.

A total of approximately 50 males and 50 females were not vaccinated and were kept at the Regional Poultry Research Laboratory as controls.

Newcastle Disease Virus Antigen for Hemagglutination Tests

A strain of Newcastle disease virus (48-49-1105) isolated in the Department of Bacteriology and Public Health, Michigan State College on May 31, 1949, from a naturally infected chicken was used to inoculate embryonating chicken eggs for the production of virus-infected

allantoic fluid to be used as the antigen in the hemagglutination tests. The virus had been originally established and cultivated in the allantoic cavity of embryonating chicken eggs through four passages and was capable of killing all inoculated embryos by the end of the second day following inoculation.

Single-Comb White Leghorn eggs obtained from a commercial hatchery were used for propagation of the virus. The eggs were incubated at 99° F. in an electric, forced-draft incubator. Embryos were 10-days-old at the time of inoculation. The eggs were candled, and an area of the chorioallantoic membrane void of large blood vessels, which was a quarter turn distant from the embryo and about 3 mm. below the base of the air cell, was selected. At this point, a hole was drilled through the shell, without piercing the shell membrane, by means of a small drill which was attached to an electric motor. A second hole was drilled through the shell over the top of the air cell. Tincture of metaphen was painted over the holes and allowed to dry. Before inoculating the eggs, a teasing needle was flamed, and a small puncture was made in the shell membrane exposed in the hole over the air cell so as to prevent leakage of the inoculum from the site of the injection because of the increased hydrostatic pressure.

The eggs were inoculated with a B-D Yale, 1 cc. tuberculin syringe fitted with a 27 gauge, 1/2 inch needle. The needle was inserted through the hole in the side of the egg to a depth of about 1/4 inch, and the inoculum was then injected. The holes in the shell were sealed with melted paraffin and then returned to the incubator.

The eggs were candled daily. Embryo mortality during the first 24 hours was considered to be due to non-specific causes and not to the action of the virus. These embryos were discarded. Embryos which were living on the second day following inoculation were selected for collection of allantoic fluid. These embryos were refrigerated overnight at 4° C. prior to collection of the fluid.

A small batch of eggs was removed from the refrigerator at a time for the collection of the infected allantoic fluid. Tincture of metaphen was applied to the shell in the area of the air cell and allowed to dry. The shell over the air cell was cracked with sterile forceps, and the shell was removed to within 5 or 10 mm. of the base of the air cell. Sterile 5 cc. syringes fitted with 20-gauge, 1-inch needles, were employed for the collection of the infected allantoic fluid. The needle was inserted into the allantoic cavity, and the fluid was aspirated into the syringe and expelled into

sterile 500 ml. Erlenmeyer flasks, which were chilled with cracked ice. Only blood-free allantoic fluid was collected. Fluid from the allantoic cavity of those embryos which were not dead on the second day was placed in a separate flask. The cotton plugs in the flasks were sealed with Para-film before freezing the fluid at — 45° C. All fluids were bacteriologically sterile.

The fluids were frozen for at least 3 days and then thawed at room temperature and dispensed in 20 ml. amounts into sterile 30-ml. screw-cap vials. These vials were then frozen until the fluid was needed. Before using, the infected fluid was thawed at room temperature and activated with formalin to a final concentration of 0.1 per cent. Formalinized virus, as indicated in the studies by Rached,[55] was stored at 5° C. for 72 hours before using to allow the virus to be completely inactivated.

Egg-yolk for Hemagglutination-inhibition Tests

Newcastle disease hemagglutination-inhibition antibodies were extracted by ethylene dichloride and ether from unincubated yolk by the method described by Schmittle and Millen.[18]

At the time of collection of the egg-yolk, the eggs were swabbed with tincture of metaphen which was allowed to dry. The shells were cracked with sterile forceps, and the egg contents were deposited in sterile Petri dishes.

With the aid of a pipette, 1.5 ml. of the un-incubated yolk material was transferred to a chemically clean 30-ml. screw-cap vial, diluted with 6.0 ml. of physiologic saline and thoroughly mixed. Two ml. of chemically pure ethylene dichloride and 1.0 ml. of reagent ether were added, and the contents of the vial were thoroughly mixed. The mixture was incubated at 37° C. for approximately 12 hours and then centrifuged at 1,000 r.p.m. for 10 minutes in an International Clinical Centrifuge.

The ethylene dichloride fraction settled at the bottom of the tube and was separated from the watery supernatant solution by a fatty ring. The supernatant fluid was removed with a chemically clean eye-dropper. This fluid, approximately a 1:5 dilution of egg-yolk, was substituted for the serum in the hemagglutination-inhibition test for Newcastle disease outlined by the U.S. Department of Agriculture.[50]

Preliminary tests indicated that the egg-yolk would have to be diluted approximately 1:160 with physiologic saline in order to bring the hemagglutination-inhibition end-points of the various samples within a readable range.

Hemagglutination and Hemagglutination-inhibition Tests

Materials:

Virus The virus antigen consisted of the blood-free pooled, Newcastle disease allantoic fluid.

Antibody The antibody was supplied in the extracted egg-yolk.

Physiologic saline 0.85 per cent chemically pure sodium chloride in distilled water.

Red blood cells Although it would have been desirable to use the blood from one chicken throughout the experiment in an endeavor to reduce variations in hemagglutination titers obtained with cells from different donors, as reported by Stuart-Harris,[56] Hirst,[42] and Miller and Stanley,[45] it became necessary to use two chickens because the original donor became ill, and the red blood cells were not suited for the test. The blood was collected in a chemically clean, sterile test tube containing 1.0 ml. of a 2.0 per cent sodium citrate solution for each 9.0 ml. of blood. This mixture was then thoroughly agitated to prevent clotting. The cells were washed three times in 10 or more parts of physiologic saline, centrifuged twice at 1,600 r.p.m. for 8 minutes and once at 1,000 r.p.m. for 10 minutes. The supernatant fluid was removed, and the washed cells were stored in the refrigerator. The stored cells were found to be suitable for use for as long as 5 to 7 days after collection. Immediately prior to use

in the test, a 0.5 per cent physiologic saline suspension of the cells was made.

Chemically clean, round bottom test tubes (75 mm. X 10 mm.) were used in the tests. Serial 2-fold dilutions of the virus from 1:5 through 1:5,280 were made in physiologic saline in a row of tubes in a wire rack. A 1:7⅛ dilution was also prepared from which serially 2-fold dilutions were made. This procedure provided 2-fold dilutions of the virus with intermediate steps and afforded a more accurate detection of the hemagglutination activity of the virus than the original 2-fold dilution of the virus. To a parallel row of tubes was transferred 0.25 ml. of each virus dilution and 0.25 ml. of physiologic saline except in the egg-yolk titration tubes in which 0.25 ml. of extracted egg-yolk was added instead of the saline. The tubes were shaken well and 0.25 ml. of a 0.5 per cent suspension of red blood cells was added. The egg-yolk control tubes contained 0.5 ml. of yolk and 0.25 ml. of the cell suspension. The control tube for the virus titration contained 0.5 ml. of physiologic saline and 0.25 ml. of cells. The tubes were shaken to mix all ingredients and were then incubated at room temperature (22° C. to 27° C.). Readings were made at 15, 30, 45 and 60 minute intervals.

To facilitate the reading of the results, the rack of tubes was placed under a fluorescent light, and

the pattern of the cells in the bottom of the tubes was viewed with the aid of a mirror under the rack.

Complete hemagglutination was exhibited as a uniform layer of agglutinated red blood cells covering the bottom of the tube and was recorded as positive (+). Where there was no hemagglutination, the sedimented cells formed a central, sharply outlined disc in the bottom of the tube and was classified as negative (-). Hemagglutination intermediate between positive and negative was classified as partial hemagglutination (±).

The titer of the virus was considered to be the highest dilution of the virus which produced complete hemagglutination. This dilution was considered to contain one hemagglutinating unit, and the number of hemagglutinating units in the undiluted virus was considered to be the reciprocal of the titer of the virus.

Calculation of Egg-yolk Hemagglutination-inhibition Titer

The titer of the virus was the highest virus-dilution before the addition of saline and cells in which + or ± hemagglutination occurred. The titer of the egg-yolk was the lowest virus-dilution before the addition of egg-yolk and cells in which — or ± hemagglutination occurred. To calculate the egg-yolk hemagglutination-inhibition titer, the titer of the virus was divided by the titer of the egg-yolk. Multiplication of the quotient

by the dilution factor of the egg-yolk gave the egg-yolk hemagglutination-inhibition titer.

RESULTS AND DISCUSSION

Results

The results of the individual tests on egg-yolk from unincubated eggs are shown in Tables III to VIII, inclusive. The average titer per pen for each experimental period and the average titer per pen for the entire experiment are shown in Table IX.

In the Control Pen, the egg-yolk hemagglutination-inhibition titers were 10 or less except at the 5th test when the titer was zero. The average hemagglutination-inhibition titer was 5 for the entire experiment.

In Pen I, the egg-yolk hemagglutination-inhibition titer for the initial test was 5,504. The titer decreased progressively to 2,560 for the 3rd test. The titers for the 4th and 5th tests were 3,925 and 11,392, respectively. The titer for the 6th test was 2,035. At the 9th and 10th tests, the titers were 9,600 and 2,400.

In Pen II, the initial egg-yolk hemagglutination-inhibition titer was 2,432. The titers decreased progressively until a minimum titer of 544 was obtained for the 5th test. Beginning with the 6th test, the titer gradually increased to 3,113 at the 8th test and decreased to 2,400 at the 10th test.

In Pen III, the egg-yolk hemagglutination-inhibition titer for the first test was 2,816. The titer

of the 2nd test decreased to 2,688. No eggs were available for the next 2 tests. The titer for the 5th test was 4,096. A titer of 2,548 was obtained for the 6th test and 2,048 for the 7th test. The titer for the 8th was 5,332 followed by a decline to 720 for the 10th test.

In Pen IV, the egg-yolk hemagglutination-inhibition titers showed greater variation than in the other pens. An initial titer of 3,328 was obtained. For the 2nd and 3rd tests, the titers were 1,920. A maximum titer of 16,992 was obtained for the 5th test. With the exception of the titer of 5,769 for the 8th test, there was a decline in titers to 448 at the 9th test. The titer for the 10th test was 1,496.

In Pen V, the initial egg-yolk hemagglutination-inhibition titer was 4,244. Other than a titer of 6,080 for the 2nd test, there was a gradual decrease in titer to 2,304 for the 5th test. A progressive increase was recorded with a maximum of 7,328 reported for the 8th test. A decrease followed with a titer of 1,712 for the 10th test.

Discussion

Eggs were tested for the presence of Newcastle disease antibodies from February 3, 1950 to April 7, 1950, and although there were fluctuations in the average weekly egg-yolk titers during the period, the over-all trend was a progressive decrease in titer.

Slight fluctuations or deviations from this normal progressive decrease in the average weekly yolk titers for the individual groups were considered to be due to the random sampling method of obtaining eggs from these groups for use in the experiment.

The sudden increases in the titers from the normal progressive decrease with the occurrence of two peaks, one at the 5th and the other at the 8th tests with a gradual decrease at the 10th test, are illustrated in Figure 1. Since these increases occurred in all pens, they could not be ascribed to differences in vaccines. One possible reason might be the seasonal effects on egg production.

The hemagglutination-inhibition test on the yolk material from the non-vaccinated chickens in the Control Pen gave an apparent diagnostic titer when the dilution, 1:160, which was used for the test on yolk material from the other pens, was employed. But when a dilution of 1:5 was used, the egg-yolk titers were not significant.

The yolk titers in the Control Pen may be considered to be due to non-specific substances. Anderson[52] reported that Burnet and his associates previously demonstrated that cells acted on by virus particles develop the ability to be non-specifically agglutinated

by most normal human and animal sera. This non-specific agglutination is associated with receptor destruction, and it may also be produced by any bacterial enzymes which can destroy receptors.

The yolk titer for the first test in Pen I, 5,504, was higher than the initial titers for any of the pens. Pen I also had the highest average yolk titer for the period of the test. All the chickens in Pens I to V were vaccinated with a vaccine which was prepared by using the same strain and amount of live Newcastle virus, but the source of the "leucosis-contaminated" vaccines differed. Since all of the vaccinated chickens received the same amount of Newcastle vaccine, the difference in the yolk titers appears to be due to the presence of both viruses in the same host.

In previous studies at the U.S. Department of Agriculture, Regional Poultry Research Laboratory, the highest incidence of lymphomatosis had been produced by inoculating susceptible chickens with a vaccine which had been prepared from infected embryo livers only. It might be assumed that the vaccines prepared from liver only for the present experiment contained the greatest concentration of the leucosis virus. Since the chickens in Pen I received "leucosis-contaminated" vaccine consisting of liver only, and the highest hemagglutination-inhibition titer was obtained in this pen, it seems

possible that the activity of the leucosis virus was attenuated. Assuming that the livers contained the greatest concentration of the leucosis virus, the use of large concentrations of inoculum might have either destroyed or saturated all receptors for which they had an affinity. The Newcastle disease virus would then have been free to initiate growth unhampered by the leucosis virus.

If the high Newcastle yolk titer is to be taken as an indication of the degree of suppression of the leucosis virus, then the lowest incidence of lymphomatosis would be expected from this group. Therefore, the extent of growth of the leucosis virus and the extent of lymphomatosis would possibly be to a degree inversely proportional to the egg-yolk titer of the Newcastle virus.

Pen II received vaccine which contained Newcastle disease virus consisting of 1/2 embryo livers and 1/2 embryo fluids. The low average egg-yolk titer, 1,612, in this pen would seem to indicate that the leucosis virus had interfered with the growth of the Newcastle disease virus. The lowered titer apparently indicated that less growth had occurred as a result of competition for metabolites. The assumption might possibly be made that since active leucosis virus was present, the chickens in this pen should show lymphomatosis. According to the theory proposed concerning the relationship of the yolk titer of the Newcastle virus antibodies and the incidence

of lymphomatosis, the highest incidence of lymphomatosis might be expected in this pen.

Pen III received commercial vaccine which consisted of the entire extra-embryonic membranes and fluids and the embryo. The lowered yolk titer in this pen also suggested interference with the growth of the Newcastle disease virus by the leucosis virus. According to the relationship which had been previously proposed for the differences of egg-yolk titers and the incidence of lymphomatosis, the average egg-yolk titer of 2,421 for Pen III, which was slightly higher than the titer from Pen II, would imply that the incidence of lymphomatosis in Pen III should be slightly less.

Pens IV and V contained chickens vaccinated with "leucosis-free" vaccine. The average egg-yolk titers were 4,097 and 3,498, respectively. These titers could be considered to be the normal for Newcastle virus as the vaccines were produced from "leucosis-free" eggs.

The incidence of lymphomatosis in these pens would be expected to be the lowest of all the experimental groups of chickens.

Data as to the actual incidence of lymphomatosis in these several groups of chickens vaccinated with "leucosis-free" and "leucosis-contaminated" vaccines are not yet completed and available for comparative studies of the possible relationship of the Newcastle disease virus egg-yolk titers and the agent of lymphomatosis.

TABLE I

Procedure for Hemagglutination

Virus Dilution

	Und.	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	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

Procedure for Hemagglutination-inhibition

Virus Dilution

	Und.	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	Control
Virus dilution ml.	. . . . .			0.25 ml. per tube					. . . . .				0
Yolk material (Dilution 1:160)	. . . . .			0.25 ml. per tube					. . . . .				0.5 ml.
Chicken Red Blood Cells 0.5% suspension	. . . . .			0.25 ml. per tube					. . . . .				0.25 ml.

TABLE III

Hemagglutination-inhibition Tests of Yolk Material at 22° C. to 27° C.

Individual and Average Weekly Egg-yolk Hemagglutination-inhibition Titers.

Pen No.	Test No.	Individual Egg-yolk H-I Titers					Average Weekly Egg-yolk H-I Titer
		1	2	3	4	5	
Control	1	10.0	7.5	0.0	20.0	10.0	9.5
	2	15.0	7.5	7.5	7.5	7.5	9.0
	3	10.0	10.0	10.0	10.0	10.0	10.0
	4	8.0	0.0	2.5	2.5	2.5	3.1
	5	15.0	15.0	7.5	7.5	7.5	10.5
	6	0.0	0.0	0.0	0.0	3.75	0.75
	7	0.0	0.0	0.0	0.0	0.0	0.0
	8	1.3	1.3	1.3	1.3	1.3	1.3
	9	0.0	10.0	10.0	7.5	7.5	7.0
	10	3.75	3.75	2.5	3.75	3.75	3.5
Average of 10 weekly egg-yolk titers							5.0

TABLE IV

Hemagglutination-inhibition Tests of Yolk Material at 22° C. to 27° C.

Individual and Average Weekly Egg-yolk Hemagglutination-inhibition Titers.

Pen No.	Test No.	Individual Egg-yolk H-I Titers					Average Weekly Egg-yolk H-I Titer
		1	2	3	4	5	
Pen I	1	640.0	5120.0	10240.0	1280.0	10240.0	5504.0
	2	7680.0	480.0	960.0	960.0	7680.0	3552.0
	3	640.0	5120.0	1280.0	5120.0	640.0	2560.0
	4	5120.0	2560.0	5120.0	5120.0	1707.2	3925.4
	5	20480.0	7680.0	640.0	20480.0	7680.0	11392.0
	6	259.0	1920.0	960.0	1920.0	5120.0	2035.8
	7	3840.0	320.0	3840.0	3840.0	640.0	2496.0
	8	2560.0	5120.0	6880.0	2560.0	10240.0	5472.0
	9	3840.0	15360.0	*	*	*	9600.0
	10	3840.0	3840.0	240.0	240.0	*	2040.0
Average of 10 weekly egg-yolk titers							4857.7

* Eggs not available at this period.

TABLE V

Hemagglutination-inhibition Tests of Yolk Material at 22° C. to 27° C.

Individual and Average Weekly Egg-yolk Hemagglutination-inhibition Titers.

Pen No.	Test No.	Individual Egg-yolk H-I Titers					Average Weekly Egg-yolk H-I Titer
		1	2	3	4	5	
Pen II	1	5120.0	640.0	2560.0	1280.0	2560.0	2432.0
	2	7680.0	1920.0	3840.0	480.0	480.0	2880.0
	3	5120.0	640.0	160.0	640.0	2560.0	1824.0
	4	160.0	1707.2	52.8	852.8	1707.2	896.0
	5	960.0	640.0	320.0	480.0	320.0	544.0
	6	960.0	960.0	480.0	480.0	2560.0	1088.0
	7	240.0	120.0	3840.0	2560.0	3840.0	2120.0
	8	208.0	2560.0	5120.0	5120.0	2560.0	3115.0
	9	960.0	240.0	240.0	1920.0	960.0	864.0
	10	10240.0	480.0	240.0	960.0	80.0	2400.0
Average of 10 weekly egg-yolk titers							1808.1

TABLE VI

Hemagglutination-inhibition Tests of Yolk Material at 22° C. to 27° C.

Individual and Average Weekly Egg-yolk Hemagglutination-inhibition Titers.

Pen No.	Test No.	Individual Egg-yolk H-I Titers					Average Weekly Egg-yolk H-I Titer
		1	2	3	4	5	
Pen III	1	2560.0	5120.0	1280.0	2560.0	2560.0	2816.0
	2	3840.0	480.0	960.0	480.0	7680.0	2688.0
	3	*	*	*	*	*	*
	4	*	*	*	*	*	*
	5	3840.0	160.0	160.0	15360.0	960.0	4096.0
	6	259.0	960.0	3840.0	3840.0	3840.0	2547.8
	7	1280.0	320.0	3840.0	960.0	3840.0	2048.0
	8	2560.0	5120.0	3408.0	10240.0	*	5332.0
	9	3840.0	1920.0	5120.0	1280.0	7680.0	3968.0
	10	960.0	480.0	*	*	*	720.0
Average of 10 weekly egg-yolk titers							2421.5

* Eggs not available at this period.

TABLE VII

Henagglutination-inhibition Tests of Yolk Material at 22° C. to 27° C.

Individual and Average Weekly Egg-yolk Hemagglutination-inhibition Titers.

Pen No.	Test No.	Individual Egg-yolk H-I Titers					Average Weekly Egg-yolk H-I Titer
		1	2	3	4	5	
Pen IV	1	320.0	5120.0	320.0	10240.0	640.0	3328.0
	2	480.0	7680.0	240.0	240.0	960.0	1920.0
	3	640.0	640.0	640.0	2560.0	5120.0	1920.0
	4	427.2	427.2	10240.0	852.8	640.0	2517.1
	5	20480.0	320.0	160.0	2560.0	61440.0	16992.0
	6	960.0	2840.0	7680.0	15360.0	960.0	5760.0
	7	480.0	1920.0	480.0	960.0	240.0	816.0
	8	2560.0	640.0	22880.0	208.0	2560.0	5769.3
	9	640.0	320.0	0.0	1280.0	0.0	448.0
	10	240.0	1920.0	5120.0	120.0	80.0	1496.0
Average of 10 weekly egg-yolk titers							4096.6

TABLE VIII

Hemagglutination-inhibition Tests of Yolk Material at 22° C. to 27° C.

Individual and Average Weekly Egg-yolk Hemagglutination-inhibition Titers.

Pen No.	Test No.	Individual Egg-yolk H-I Titers					Average Weekly Egg-yolk H-I Titer
		1	2	3	4	5	
Pen V	1	2560.0	10240.0	2560.0	640.0	5120.0	4224.0
	2	15360.0	7680.0	3840.0	960.0	2560.0	6080.0
	3	5120.0	640.0	5120.0	427.2	1280.0	2517.4
	4	160.0	1707.2	3412.8	5120.0	1707.2	2421.4
	5	2560.0	1920.0	640.0	1280.0	5120.0	2304.0
	6	7680.0	960.0	1920.0	1920.0	2840.0	3264.0
	7	7680.0	240.0	1920.0	*	*	3280.0
	8	320.0	20480.0	5120.0	20480.0	10240.0	7328.0
	9	2560.0	3840.0	1280.0	640.0	960.0	1856.0
	10	240.0	3840.0	1920.0	1920.0	640.0	1712.0
Average of 10 weekly egg-yolk titers							3498.6

* Eggs not available at this period.

TABLE IX

Hemagglutination* Inhibition Tests of Yolk Material at 22° C. to 27° C.

Average Titer per Pen for Each

Experimental Period and the Average Titer per Pen for the Entire Experiment

Pen No.	Average Weekly Egg-yolk H-I Titers										Average H-I Titer per Pen for entire Experiment
	1	2	3	4	5	6	7	8	9	10	
Control	10	9	10	3	11	1	0	1	7	4	5
Pen I	5504	3552	2560	3925	11392	2035	2496	5472	9600	2040	4858
Pen II	2432	2880	1824	896	544	1088	2120	3113	864	2400	1808
Pen III	2816	2688	*	*	4096	2548	2048	5332	3968	720	2422
Pen IV	3328	1920	1920	2517	16992	5760	816	5769	448	1496	4097
Pen V	4224	6080	2517	2421	2304	3264	3280	7328	1856	1712	3498

* Eggs not available at this period.

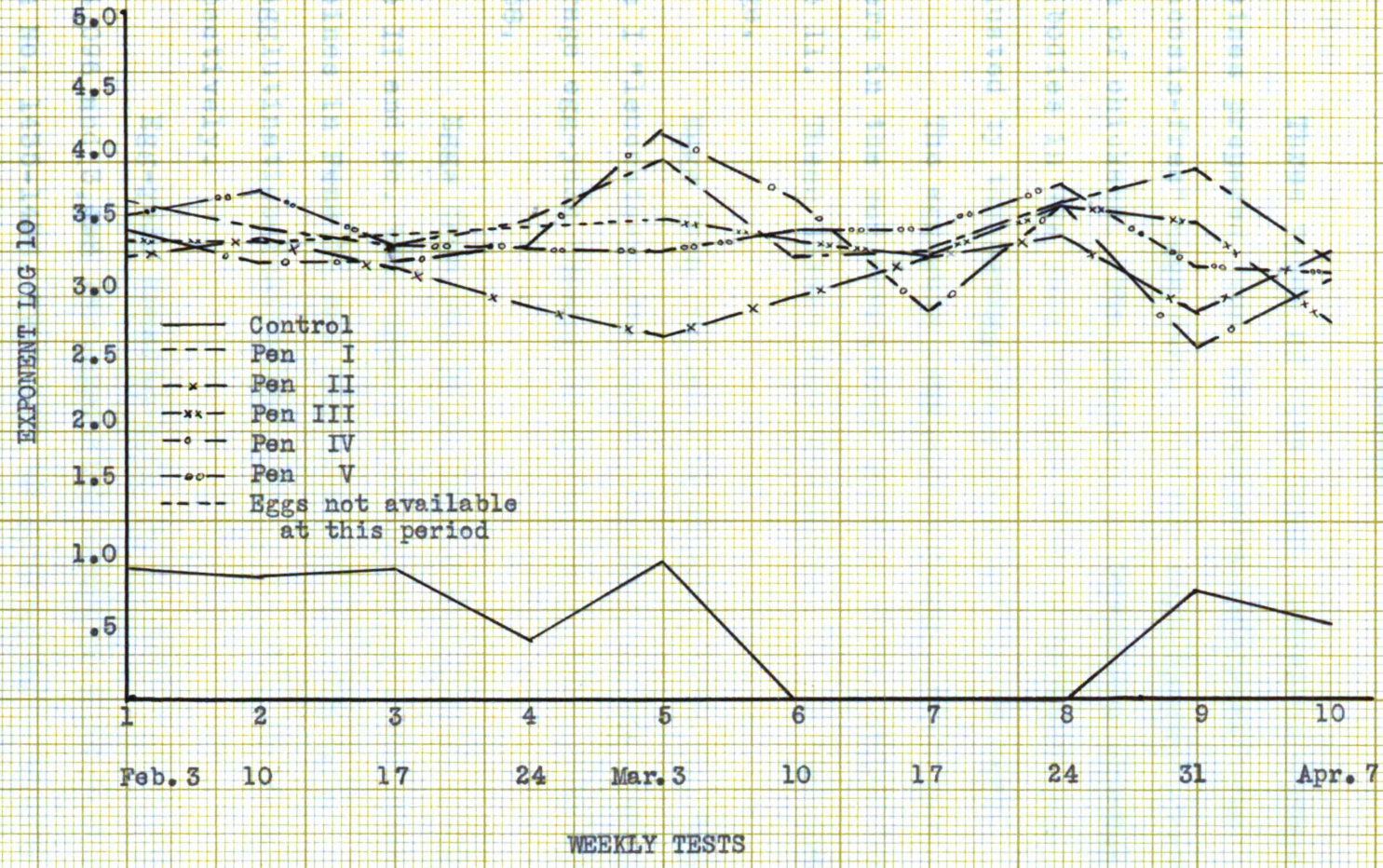

Fig. 1. Average weekly egg-yolk hemagglutination-inhibition titers for experimental period.

SUMMARY

Three types of live-virus Newcastle disease vaccines prepared from "leucosis-contaminated" and "leucosis-free" eggs were used for experimental vaccination of chickens. The presence of Newcastle disease antibodies in the yolk of eggs from vaccinated hens was evaluated by the hemagglutination-inhibition test.

The egg-yolk hemagglutination-inhibition titers in the non-vaccinated Control Group ranged from 0 to 11. These titers were considered to be the normal range.

Eggs from chickens vaccinated with No. 707, Type I "leucosis-contaminated" vaccine in Pen I had an average egg-yolk hemagglutination-inhibition titer of 4,858.

Eggs from chickens vaccinated with No. 707, Type II and No. 707, Type III "leucosis-contaminated" vaccines in Pens II and III, had average egg-yolk hemagglutination-inhibition titers of 1,801 and 2,042, respectively.

Egg-yolk hemagglutination-inhibition titers of 4,096 and 3,498 for eggs from chickens vaccinated with No. 1435-1436, Type III "leucosis-free" vaccine in Pens IV and V might be considered normal for Newcastle disease virus as these vaccines were produced from "leucosis-free" eggs.

Fluctuations in average weekly egg-yolk titers of eggs from the different pens during the course of the experiment were considered to be due to the random sampling method of obtaining eggs from each group.

The seasonal effect on egg production may have contributed to certain variations in egg-yolk titers during the experiment.

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