HEMAGGLUTINATION-INHIBITION TEST

AS APPLIED TO THE CONGENITAL IMMUNITY AND DIAGNOSIS OF

NEWCASTLE DISEASE

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Robert H. Leonard

TABLE OF CONTENTS

INTRODUCTION

Since the identification of Newcastle disease in baby chicks in California in 1944, outbreaks have been observed and identified in every state. The high morbidity and mortality rates of the malady have placed it in a position of great interest.

Development of the diagnostic procedures have been largely based upon the hemagglutination test as first described by Hirst with the influenza virus. The phenomenon was at first considered specific, but it is now known that the reaction is very complex and several substances have been found which react or interfere with some phase of the test. The presence of antibody in blood serum has been detected by measuring the inhibition of the hemagglutination test.

It has been known for some time that chicks hatched from immune hens carry a certain refractivity to infection with the virus for about the first thirty days. Recently, outbreaks of the disease have been observed in chicks from supposedly immune hens. These irregularities, as well as difficulty in obtaining the expected protection from a vaccination of young birds, have led to renewed interest in the importance of the congenital immunity involved. The impracticability of taking blood samples from laying hens to determine their immune status has given rise to the development of a hemagglutination test using the egg yolk. This test, when adopted, will give the poultryman and hatcheryman not the immune status of the hen only, but also the amount of immunity that she confers to her offsprings. From this data, vaccination programs can be more easily planned.

The present work has served to give a better knowledge of the hemagglutination test in general and to correlate the results of the several procedures. In one group of birds the status of the immunity was unknown; here the test

was used to detect antibody. Those hens had no previous history of Newcastle disease but were observed to develop a respiratory condition which spread through contest pens, possibly originating in a pen of birds which had been vaccinated with live virus.

The immense economic aspects of Newcastle disease has provided a great stimulus for studies. The general accelerated response to virus work, especially with regard to vaccines, immunizations, congenital immunity, and diagnosis undoubtedly will lead to a more complete understanding of other virus reactions.

In view of these facts, this experimental work was considered worthwhile.

REVIEW OF LITERATURE

"A highly diffusible and fatal infection of poultry" was the description given by Kraneveld in 1926 to a disease in the Dutch East Indies, which is now generally termed Newcastle disease. Doyle (1927) in the same year observed this disease at Newcastle-on-Tyne, England, named it after that place and demonstrated the filterability of the causative agent. The first recognition of this malady in the Western Hemisphere was made in California in 1944 (Brandly, et al., 1946; Beach, 1944). The disease undoubtedly existed (Beaudette, 1946) in the United States on a wide scale long before this identification was made.

Burnet (1942) and Lush (1943) observed that the Newcastle virus exhibited the agglutination of certain red blood cells. The use of this phenomenon in identification and diagnosis of the disease makes a review of the pertinent facts of particular interest.

Hirst (1941) first demonstrated the agglutination of red blood cells by the influenza virus and the inhibitive action of immune serum. This reaction has since been known as Hirst's phenomenon.

He observed that when infected allantoic fluid of a chick embryo was mixed in a test tube with washed normal adult chicken red blood cells, an agglutination phenomenon occurred. The red cells sedimented rapidly and formed a characteristic ragged granular pattern on the bottom of the tube. If the red cells were added in the test tube to the allantoic fluid from an uninfected chick, only slow sedimentation of the red cells occurred with no aggregation; and in settling out, the cells formed a sharp-edged round disc in the bottom of the tube. He also noted that when normal chick embryo red cells were added in sufficient numbers to infected allantoic fluid and

allowed to settle out, over 99 per cent of the virus present in the infected fluid disappeared from the supernatant fluid.

Further experimentation showed that when the allantoic fluid infected with the virus was centrifuged (45 minutes at 11,500 r.p.m.) the "titer" of the supernatant in terms of the agglutinating capacity dropped approximately four times. This fall in agglutinating power was consistent with the expected drop in virus titer as demonstrated by previous tests in mice with the same fluid, which showed that 70 to 90 per cent of the virus sedimented. If, instead of infected allantoic fluid, the supernatant from centrifuged ground mouse lung infected with PR8 mouse passage virus was used, the added red cells were agglutinated in a dilution as high as 1:5000 (final concentration of mouse lung). When influenza virus ferret antiserum (PR8) in dilutions as high as 1:1024 was mixed with allantoic fluid infected with the homologous virus, the agglutination phenomenon was inhibited. The inhibition was specific, that is, influenza B ferret antiserum in dilutions as low as 1:8 failed to inhibit agglutination of red cells by fluids containing influenza A virus. Such inhibition also occurred with human serum, titrations were made of acute and convalescent serum from proven cases of influenza A. Dilutions of serum were mixed with constant amounts of WS infected allantoic fluid; then a constant amount of chick red cell suspension was added, and the agglutination was read in one hour. The change in titer of this agglutination-inhibition substance following infection appeared to be of the same order of magnitude as the rise in the patients' neutralizing titer against PR8 virus as determined by the mouse.

Following the original discovery by Hirst with the influenza virus subsequent observations of the same reaction with certain other viruses, including

the Newcastle virus (Burnet, 1942), were made.

This "in vitro" virus-cell reaction has given an excellent opportunity to study the factors involved and the relationships existing, in an effort to better understand the relationships "in vivo".

Hirst (1942a) showed that hemagglutination of the virus was associated with the adsorption of the virus to the cell surface which was subsequently followed by progressive reliberation of the virus. In the process, the red cell lost its capacity to adsorb or be agglutinated by fresh virus.

The curves of adsorption and elution of virus hemagglutinins suggests (Hirst, 1942a) that the interaction of hemagglutinin and cell occurs in two phases: first, a combination producing agglutination, and second, some alteration of the cells accompanied by a separation of the modified cells and the agglutinin. The modification of the cells in the second phase renders them incapable of combining with more agglutinin, while the released agglutinin is apparently unchanged.

The analogy of this reaction with the interaction of enzymes and substrates was brought out by Hirst (1942a). It is the generally accepted view that the first stage in enzymatic reactions, at least in most cases, is the combination of enzyme and substrate. Then the substrate is chemically changed, whereupon the enzyme and the altered substrate dissociate, and the enzyme is free to adsorb and alter more substrate. It is clear that if one were able to make a measurement of the amount of free enzyme present during this process, the amount of free enzyme should be low in the initial stages after combination had occurred in the presence of an excess of substrate. In the later stages of the enzymatic action when the substrate has been largely used up, free enzyme should appear again in quantity approaching the initial concentration, since there is insufficient substrate to combine with all of the

enzyme present.

In this analogy the agglutinin corresponds to the enzyme, which is not used up, while the substrate corresponds to the substance at the receptor point on the red cell, which is destroyed during the process of agglutination, rendering the cell incapable of further adsorption. The sensitivity of the hemagglutinin to heat (Hanson, 1949; Lauffer and Scott, 1945, 1946a, 1946b) and to formalin (Salk, 1946) is consistent with a substance of protein nature. The cellular substrate, on the other hand is very resistant to heat, and this stability suggests that it may be a non-protein substance.

A direct attempt to define this cell component has been made by Bovarnick and Burgh (1947), who succeeded in preparing from large amounts of human erythrocytes a lipid-polysaccharide extract capable of inhibiting the hemagglutinating action of the same viruses for those cells. The effect is presumably due to the competitive combination of the extract with the virus.

The inhibition reactions thus far tested seem to demonstrate specificity corresponding to that shown by the viruses in their hemagglutination reactions (Bovarnick and Burgh, 1947). Prolonged contact of the virus and inhibitor at 25° C or 37° C shows that the interaction between the two results in progressive inactivation of the inhibitor, evidenced by reappearance of increasing hemagglutinative activity in an inactive mixture. The similarity of this reaction with Hirst's (1942a) description of the reversal of hemagglutinating activity lends support to the notion that the inhibitor is a derivative of, or identical with, the cell receptor.

The simplest explanation of the actual above agglutination of cells in the presence of the agglutinin is to view the latter as forming a bond between the cells. If the agglutinin particles were bi- or trivalent and there were multiple receptors on the red cells, one could visualize how a network or

clump of cells could be built up. The main evidence for this view is the fact that the agglutinin adsorbed and the agglutinability of the cells are parallel phenomena, (Lind, 1948).

If hemagglutinin and cell sensitizing agent are both activities of the virus particles themselves, their apparent dissociation by replaced treatment with red cells calls for some explanation. It is simplest to assume that conditions are similar to those found by Anderson (1947) for Newcastle disease virus, viz., that the cell sensitizing agent is essentially the fraction of virus particles which is capable of adsorption but not of subsequent elution. The larger fraction of the virus particle population is composed of units which can be repeatedly adsorbed on red cells, cause destruction or distortion of receptors and be subsequently eluted.

A suspension of human red cells, coated with virus by Burnet's method (1946), acts as an indicator substance. The action can be described as follows: on each cell there are adsorbed several virus particles. Owing to the weakness of their eluting system, these particles are firmly held and protect the corresponding cell receptors from either contact with virus hemagglutinin or with a receptor destroying enzyme (RDE). Despite the absence of an eluting system, these particles can still unite with virus receptors on normal red cells, so causing agglutination when treated and normal cells are mixed. When immune serum is added to treated cells, antibody forms a bridge between adsorbed particles on different cells and agglutination occurs. A suspension of treated cells is stable because all receptors unoccupied by unelutable particles have been rendered ineffective to hold virus by the activity of active hemagglutinin. The effect on the receptors is only partial (Burnet, McCrea and Stone, 1946) and by lowering the temperature, sufficient union with the damaged receptors on other cells can occur

to induce instability of the suspension. The effect of antibody is to allow union between two cells by means of the attached unelutable virus particles and hence agglutination.

Evidence of the serological identity of the hemagglutinin (HA) and the cell sensitizing agglutinin (CSA) is provided by the finding that the titer of antihemagglutinin is proportional to that of sensitized cell agglutinin in immune serum and serum from vaccinated individuals, as shown by Burnet (1946a).

The strikingly different results obtained in a number of experiments, according to whether fowl or human cells were used to measure the hemagglutinative activity of treated virus, are probably all referable to a firmer adsorption of the virus to fowl cell receptors than to those of human cells (Lind, 1948). In general, any agent or treatment that tends to inhibit the action of the virus will appear disproportionately effective against human as compared with fowl cells, (Lind, 1948).

Lind (1948) stated that at 4° C union between virus and fowl cell is firm enough to ensure breakage of the forces producing aggregation of virus particles. With human cells at that temperature, the virus-cell union usually breaks before the virus-virus union within the aggregate, and only at higher temperatures do the aggregates automatically disperse in the presence of susceptible human cells. Under optimum conditions at 37° C, titers produced with the two types of cells are equivalent. Under the influence of heat, formalin or aging, she found the diminished adsorptive power of the virus is first shown against human cells, and if non-specific inhibitors are included in the system they too are apparently more effective when human cells are used as indicators of their effect on the virus. The virus thus, must be of full normal character to make a satisfactory union with the human cell receptor. Any deviation from the normal greatly weakens or abolishes the union.

Union with fowl receptors has a higher tolerance for minor structural changes in the virus and for the presence of weak inhibitors in the system. This is further borne out by the behavior of freshly isolated strains of virus and of strains which have been intensively passaged in eggs, both of which show a variable capacity to agglutinate human cells although the fowl cell agglutination titers may be uniformly high (Lind, 1948).

It is interesting to note the close resemblance between the mumps virus and the Newcastle virus as brought out by Anderson (1947). Both viruses have the capacity to sensitize red cells, rendering them specifically agglutinable by immune sera and capable of causing the agglutination of added normal cells. In both cases there is evidence for aggregation of virus particles in infected fluids.

Hirst (1942c) has shown that the infective agent in a virus suspension was adsorbed and eluted in a manner similar to that of the hemagglutinin, and that there is a close association between these two types of activity. Whether these two types of activity are the action of two agents occurring on the same particle or on different particles, and whether these two forms are completely separable was not mentioned by him. So far, the agglutinin may be obtained without the virus activity, but there is no evidence that the agglutinin can be inactivated without also inactivating the virus (Hirst, 1942c).

Hirst (1942c) also stated that it seems more likely that the agglutination of red cells by virus hemagglutinin may have some counterpart in natural infection of susceptible cells with the virus. While there is no evidence at all concerning this problem at present, he stated that it may be that susceptible cells possess a surface receptor similar to that of the red cells and that in infection this substance is altered by the hemaggluti-

nin, thus injuring the cell membrane and providing a point of attack for the infective particle.

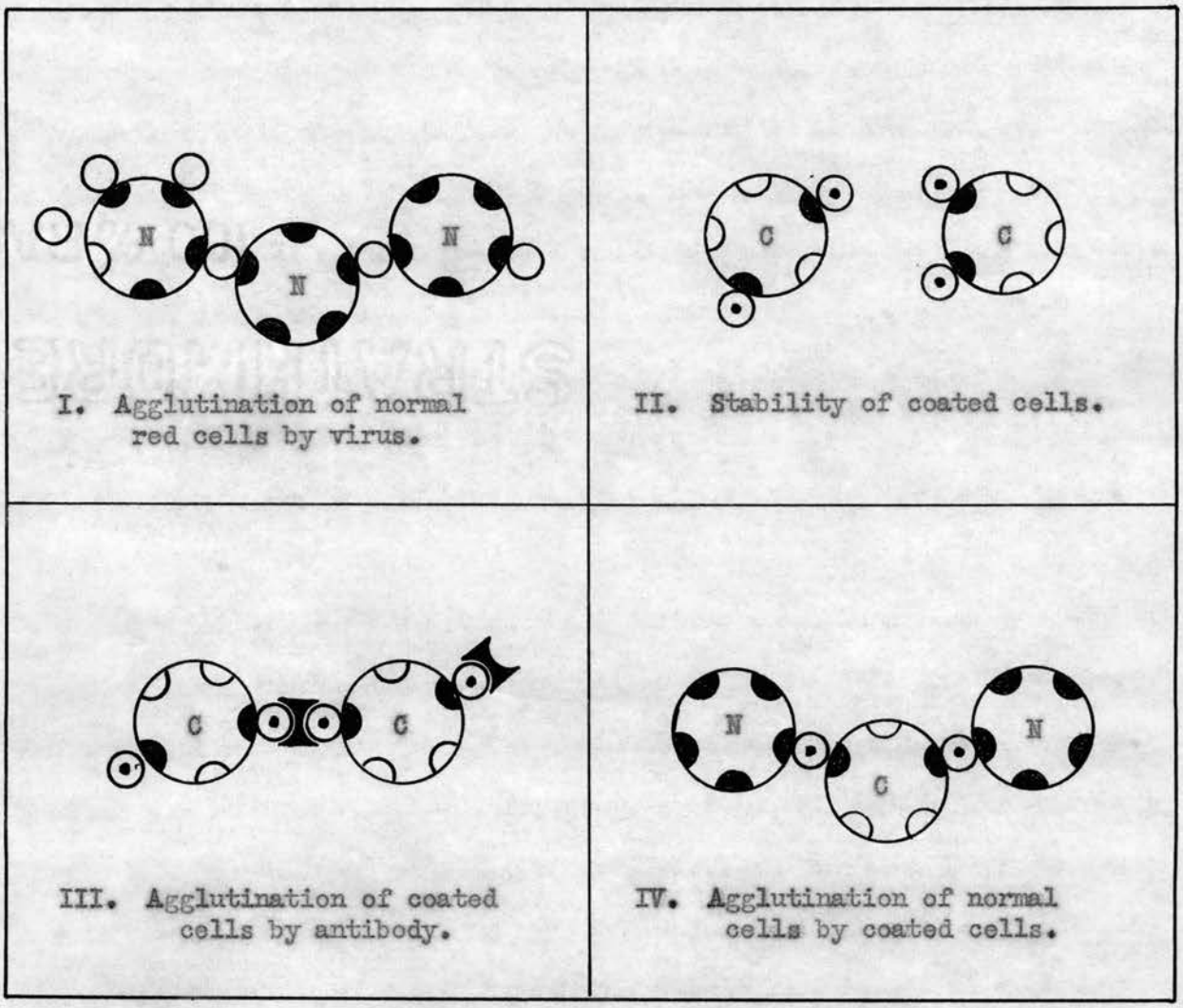

Figure 1. Diagram to indicate the reaction of virus with red cells. The representation is purely schematic; large circles represent red cells, small ones virus particles, a central dot indicating that they are unelutable. Effective receptors are shown as black semicircles, modified ones by open semicircles. [1]

Francis (1947) reported that influenza B virus upon heating to 55° C became extremely sensitive to inhibition by normal serum. With this indicator

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[1] Lind, Patricia. 1948. Nature of mumps virus action on red cells. Austr. J. Exp. Biol. & Med. Sci., 26:93-106.

strain a number of other substances, including egg albumen (Ianni and Beard, 1948), have been shown to react as "Francis inhibitors".

Receptor destroying enzymes (RDE) have also been discovered, Anderson (1948) reported the action of the cholera vibrio enzyme as possessing this RDE characteristic.

Hirst (1948) has demonstrated the presence of a substance in plasma that inhibits the agglutination of red cells by virus which is distinct from antibody. He also has shown a similar substance from the stroma of fowl red blood cells.

Burnet (1948a) concluded that the viruses are adsorbed by all cell receptors, the essential component of which is a mucopolysaccharide.

The adsorptive power of the receptor for virus and its susceptibility to inactivation by virus enzyme are considered by Anderson (1948a) to be closely related, because of the action of any of the effective enzymes the receptor becomes progressively incapable of adsorbing an increasing number of viruses, so giving the phenomena of the receptor gradient. He explains that as each unit of enzymatic action is completed, the enzyme concerned is freed for action on an adjacent susceptible configuration. Under the action of Brownian movement and the various adsorptive forces concerned, the virus particle can be regarded as "browsing" over the patch until such a condition is reached that the adsorptive forces are no longer strong enough to maintain it in contact with the receptor. When the action of a certain strain of virus has ceased, there are still many available substrate configurations, but only viruses more readily adsorbed than the previous ones are capable of further reducing their numbers (Anderson, 1948a).

Bovarnick and Burgh (1947) consider that two such complex surfaces as those of the virus particle and red cell probably demonstrate qualitative

differences in adsorptive affinity revealed in the receptor gradient.

The affinity of red cells for viruses differ according to the source of the cells, and for easy detection of inhibition other than antibody, the red cells should possess a relatively low affinity for the virus used. Anderson (1948b) also stated that the affinity between inhibitor and virus will naturally depend upon both components, and treatment of either will modify the reaction.

Beach (1943) made the first attempts to apply these phenomena to the diagnosis of Newcastle disease. In view of the complicated reactions and the numerous factors involved it was not until 1948 that a successful application of the hemagglutination-inhibition test was reported (Beach, 1948). Previously, all diagnoses were necessarily made by isolation and identification of the virus (Brandly, et al., 1946) and by the serum neutralization procedure.

Isolation of the virus has not been completely successful. The difficulty encountered in the selection of tissue infected with the virus has been variously reported (Beaudette, 1943; Brandly, et al., 1947). The saliva, lung, spleen, bone marrow, brain, spinal cord, egg yolk, crop, intestinal content, feces, and blood have all been reported by them as sources of isolation. Their experience has shown that none of the tissues are regularly infected and no pattern of the location of the virus in tissue to the course of the disease could be determined. Osteen and Anderson (1948) reported that in less than fourteen per cent of the total attempts is virus recovery made. The presence of so few laboratories with adequate means of virus isolation and the time required for such tests rendered them impractical except from an epizoological standpoint, as brought out by Brandly, et al. (1946b).

The need and desirability of using only serologic procedures for the diagnosis of Newcastle disease were related by Osteen and Anderson (1948). Since the ultimate remedial procedures are dependent to a great extent upon a rapid method of diagnosis, they determined by experiment that the hemagglutination-inhibition test gave quicker results on a larger number of birds than did the serum neutralization test. The simplicity of the serum neutralization test (SN) has made laboratory workers reluctant to adopt the more complicated newer methods. The SN test as related by Osteen and Anderson (1948) consists simply of inoculating ten to twelve day chicken embryos with a standardized virus suspension and the unknown serum in definite dilutions simultaneously, the presence of antibody being manifested by the neutralization of the virus and consequent failure of the infection to occur. They state that the value of the SN test as a diagnostic aid is dependent upon its accuracy in estimating the diagnostic antibody level and upon the reliability of positive findings as an indication of exposure to Newcastle virus. They also have shown that in no case was the neutralizing titer higher than 100 embryo m.l.d.'s in a composite sample from flocks with a negative history. In flocks where positive diagnosis of Newcastle had been made, neutralization titers of at least 1000 embryo m.l.d.'s were reached. The hemagglutination-inhibition diagnostic titer was observed by them to be obtained earlier in the course of the disease than was the diagnostic serum neutralization titer. Using 10^3 as the diagnostic SN titer and 80 as the diagnostic HI titer, they reported that the HI test (Beach, 1948) was equally as reliable and accurate, if conducted with care, as was the SN method.

Beach (1948) regards the HI test as sufficient for the identification of Newcastle disease in chickens from areas in which the disease is known to occur. Due to the complexity of the reaction and the multitude of uncon-

trollable factors involved in the hemagglutination-inhibition test, it alone should not establish the presence of Newcastle disease in an area previously believed to be free of the disease (Beach, 1948). He also considers the handling of the formalized virus which is suitable for the HI tests as a decided advantage over the every day use of the live virus used in the SN method.

In the standardization of the HI test for diagnostic procedure, it has been found that certain factors must be carefully controlled to give consistent results (Beach, 1948).

Although a virus strain is highly virulent, its suitability as a diagnostic culture is not always evidenced (Beach, 1948). Usually a highly infective virus will give a comparative HI titer, but often a high degree of variation in the strain is observed, giving irregular and unsatisfactory hemagglutination readings. Beach (1948) states that the term strain is used here to indicate virus isolated from different sources and does not indicate necessarily immunologic or other differences in their nature. As pointed out by him, a strain of high infectivity very often does not readily adapt itself to embryo culture sufficiently to give a concentrated virus suspension of the harvested embryo fluids. The same results were obtained by Brandly, et al. (1946) and it was also found that occasional cultures kill the embryo before a concentrated growth has occurred. They reported that following inoculation upon the serosa, the allantoic-amnionic fluids were not hemagglutinative, or only slightly so, while with rare exceptions the fluids from eggs infected fatally via the allantoic, amnionic, or the intravenous routes were active in this respect. While a virus strain isolated locally usually gave a more homologous antigen for the test, other characteristics must be determined before the selection is made.

Chicken red blood cells have been shown by Burnet (1942), Clark and Nagler (1943), and McClelland and Hare (1941) to be the most satisfactory indicators for the test. The inability of the red cells from a horse to agglutinate with the Newcastle virus has been used by Brandly, et al. (1946a) to distinguish it from the often confused fowl plague and the influenza A virus. Clark, et al., (1943) considers the human red cells as more sensitive, although probably less available. Table 1 shows the reactions as determined by Brandly, et al., (1946) as to the suitability of red blood cells from various sources.

TABLE 1

Data on Agglutination of Erythrocytes of Different Species by Newcastle.[2]

Species	Lush	Burnet*	Clark & Nagler*	H.R.L.**
Chicken	+ + +	+ + +	+ + +	+ + + +
Horse		−	−	−
Cow			+ + +	+ +
Sheep			+ + +	−
Guinea Pig		+ + +	+ + +	+ + + +
Monkey (rhesus)				−
Monkey (irus)		+		
Dog				+ + + +
Cat			−	−
Man		+ + +	+ +	+ + +
Rabbit		−	+	

H.R.L. - Huntington Research Laboratories.

* - Consider complete agglutination as 3 plus, whereas this result is indicated as 4 plus by the other work cited.

** - Other strains showed less positive results.

All workers are in complete agreement on the fact that variability exists between individuals within a species as to the suitability of the red cells

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2 Brandly, et al., 1946. Isolation and identification of Newcastle from infected chickens. Am. J. Vet. Res., 7:289-306.

(Brandly, et al., 1946; Clark and Nagler, 1943; Burnet, 1942; Lush, 1943). Certain birds were found to provide red cells of abnormally high hemagglutinative stability; others have failed to agglutinate at all. With regard to the number of red cells used, Beach (1943) recommended a one per cent suspension of washed cells, although Hirst (1941) considered a two per cent suspension more satisfactory. Brandly, et al., (1946) stated that irregular results are often due to the use of a too heavy suspension of red cells and recommended a one per cent suspension.

The presence of the Newcastle in the blood of a bird, either from vaccination or natural infection usually has been found to render the cells unsuitable for use (Lush, 1943).

The temperature and time required for hemagglutination by the Newcastle virus are still points of disagreement. Beach (1943) completely abandoned the use of the hemagglutination test with Newcastle virus in 1943, after all of his efforts had given negative results. Later in that same year, renewed attempts revealed the time of reading the results had been incorrect. In these early trials, readings were made after one hour at room temperature according to the Hirst (1942) technique, after two hours in the refrigerator according to the Burnet technique (Burnet, 1942), or after overnight in the refrigerator according to the technique of Krueger, et al. (1943). Later Beach found that sedimentation of agglutinated cells takes place within thirty minutes at either room or refrigerator temperatures and that the agglutinated cells slide to the lowest part of the curvature of the bottom of the tube so rapidly that a test read as positive in thirty minutes might appear completely negative in an hour or so after the test was set up.

The results of Doyle (1927) indicated that Newcastle virus in the mouth exudate of infected birds had a relatively broad pH stability range. The

work of Hanson (1949) and that of Moses (1947) both demonstrated that a pH of 7.2 is the most stable hydrogen ion concentration for the virus, but very little change in its activity was noted between pH 4.0 and 8.0.

Lowell, et al. (1948) were unable to demonstrate that the salt concentrations of the substances involved in the tests would materially alter the results under normal conditions. They noticed the greatest change in the hemagglutinative activity when the calcium level was reduced, but these changes were not significant.

The HI test differs from many serological tests in that the titer of the antigen is determined and the antibody titer is expressed in terms of the inhibition of the antigen. The Bureau of Animal Industry[3] has standardized the test somewhat in specifying the virus suspension to be such that intramuscular injection of chicks, 30 to 60 days old, with 0.1 cc of dilutions of it up to 1:1,000,000 or higher produce fatal infection. They consider 80 as a diagnostic titer, that is, the suspension of virus, red cells and unknown serum requires 80 times as many viruses to bring about agglutination of the red cells as is required for the reaction in the absence of the serum.

In reading the results of an HI test, the method of Beach (1948) and Salk (1944) have been largely adopted. When there is no agglutination, the sedimentation of the cells forms a small disc of gradually increasing size at the lowest point of the curvature of the bottom of the tube. When agglutination occurred, the sedimenting cells formed either a thin uniform blanket covering the entire bottom of the tube (+ + + +); a thin blanket with an irregular outline covering about two-thirds of the bottom of the tube (+ + +);

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[3] Special Mimeograph on the diagnosis of Newcastle disease from the Pathological Division of the Bureau of Animal Industry. August 15, 1946.

a small central disc surrounded with a granular area of agglutinated cells (++); or a larger central disc with a slightly granular periphery (+). The highest dilution of the virus which gives at least a two plus (++) agglutination of red cells is termed the hemagglutination titer of the virus.

Following the standardization and general acceptance of the reliability of the HI test with blood serum, Schmittle (1948) developed a procedure whereby the test could be applied to fresh egg yolk.

Studies on the transmission or "inheritance" of immunity in birds was first reported by Klemperer in 1893, he found that the yolk but not the white, of eggs laid by hens actively immunized against tetanus gained antitoxic properties. Ramon (1928) verified this work.

Later Dziergowski (1901) reported very similar findings with diphtheria immunized chickens. He concluded that the antitoxin may pass from the serum of the hen to the egg cell during maturation of the graafian follicle. Also, that the circumstance of "immunity inheritance" was due to passive congenital acquired immunity which was passed from the globulin of the dam's serum to the embryo unchanged.

Jungherr, et al. (1948), in work with infectious bronchitis and Brandly, et al. (1947) with Newcastle disease have shown that the passive acquired congenital immunity which is demonstratable in the yolk is not present in any other portion of the embryonating egg in the early stages of incubation. They observed that the antiviral activity appears in increasing amounts in the tissue of the embryo beginning about the tenth day of incubation and reaching a maximum by the sixteenth to eighteenth day. There was a subsequent decrease noted in the immune substances in the yolk during this same period.

Jukes, et al. (1934) and Fraser, et al. (1934) determined the livetin fraction of the egg yolk and the serum globulin of the hen as the fractions

associated with the antibodies. The serum antibody titer and resistance of the newly hatched chicks were found to diminish rapidly and disappear completly at three to four weeks of age in the case of diphtheria (Ozawa, 1936), fowl plague (Hallauer, 1936), and Newcastle (Brandly, et al., 1946). The congenital immunity which protects newly hatched chicks has been shown to prevent satisfactory development of active immunity by Hallauer (1936) and Brandly, et al. (1946).

Brandly, et al. (1946) reported the Newcastle antiviral activity of egg yolk. Their work was mainly concerned with the possible interference of this activity with virus cultures grown on egg embryos and with the serum neutralization diagnosis. They concluded that no marked interference was evident when embryos of less than 15 days incubation were used. The antibody being transferred from the yolk to the tissues of the embryo primarily in the last four to six days of incubation. They found the titer of the antibody of a newly hatched chick was almost identical to that of the blood of its dam.

The HI test as applied to egg yolk by the method outlined by Schmittle (1948) is considered by Brandly, et al. (1947) as a good indication of the immunity of the dam to Newcastle. They found that the titer of the yolk was somewhat lower than that of the blood serum of the hen and therefore recommend that allowances be made. The practical aspect of this test for laying hens in contrast to the blood serum HI test is reported by many workers. Brandly, et al. (1946) and Schmittle, et al. (1948) believe the less equipment and labor needed, and less decrease in egg production, as well as the ease in handling, shipping and storage of the sample to be tested more than offset the disadvantages encountered. The test can of course, only be run on laying hens from which absolute identity of each hen's egg can be made. They consider the test of no value as a diagnostic aid but serving as an indicator

to the hatcheryman as to the expected resistance of chicks to infection during the first month.

Brandly, et al. (1948a) found the presence of the virus in the yolk of eggs from hens recently vaccinated with live virus or during outbreaks of the disease, this would render the HI test on the yolk at this time inaccurate.

EXPERIMENTAL WORK

A. Materials and Methods

1. Virus culture and standardization: The virus strain used in these experiments was the #7 Oklahoma strain, isolated June 16, 1948 from the larynx of a bird suspected of Newcastle disease. Over a period of time, several strains of virus were isolated from different birds by grinding the tissue and suspending it in saline, this was followed by simultaneous inoculation of 12 day embryos and 4-6 week chicks. Aseptic procedures were used in all work as far as possible and small amounts of penicillin were added to the suspension to reduce bacterial contamination.

The strain #7 was successfully isolated from the above eggs and birds free from contamination and proved to have a very high hemagglutinative titer. Consequently, this strain was selected for the laboratory diagnosis strain.

A suspension of the virus was sent to the Bureau of Animal Industry for verification of the identification. In a report received August 18, 1948, the Newcastle virus identification was verified and the disease was officially recognized in Oklahoma.

The virus was stored in a frozen state, and the hemagglutinative titer was checked at intervals. At no time was the titer of the virus less than 1:320.

All virus suspensions used in the experimental work were grown on 10-12 day embryos from non-immune hens. The virus was harvested as blood-free allantoic-amnionic fluids when 50% or more of the inoculated embryos were dead, as determined by candling the eggs. Bacterial sterility was regularly checked each time inoculations were made. No virus stored longer than thirty days was used in the hemagglutination-inhibition tests.

Virus suspensions used in the hemagglutination and hemagglutination-inhibition tests were checked and used only if they satisfied the following Bureau of Animal Industry recommendations. Intramuscular injections of 0.1 ml. of 1:1,000,000 dilutions of the virus suspension must according to these recommendations produce a fatal infection in susceptible 30-60 day old chicks. In order to facilitate the standardizations, the original virus suspension was checked as above and titered against a 1:5000 titer antiserum and against the hemagglutination titer of a California strain virus, both of which were obtained from the Bureau of Animal Industry. Subsequent virus suspensions were demonstrated to show the same titer against the antiserum and the California strain virus. At the conclusion of the experimental work the virus was again checked with 30-60 day old chicks to confirm its infective titer.

The suspensions of virus used in demonstrating the neutralizing antibody in 15-17 day embryos was standardized on the basis of the minimum infective embryo inoculation. Eggs from hens with no history of Newcastle disease and no Newcastle antibody as demonstrated by the serum HI test were inoculated on the 15th day of incubation with 0.01 ml. of virus dilutions increasing tenfold throughout the series. The virus suspension was standardized to contain one infective embryo dose in 0.01 ml. of a 1:100 dilution. Embryos from birds showing a diagnostic HI serum titer were demonstrated to resist a challenge of 0.01 ml. of a 1:10 dilution or more.

2. Eggs and serum: The eggs used for culture and controls were from an isolated flock of hens having no history of Newcastle disease. Blood serum and yolk HI titers were determined, indicating no bird with more than a 10 hemagglutination-inhibition titer.

Eggs referred to as "immune" were obtained from a pen of birds with a

history of Newcastle and live virus vaccination. These hens showed an individual HI titer of 80 or more and the pooled serum of the flock a titer of 160.

All eggs used were from mated flocks. Incubation of the eggs was carried out at 99° F. and 85-86% relative humidity in a Humidare Rotary electric incubator.

All inoculations were made by route of the allantoic fluids.

Eggs and serum from birds of an unknown status were from a flock which had been affected with a mild respiratory condition which clinically resembled Newcastle disease, or had been vaccinated at some time.

Positive Newcastle antiserum with a titer of 1:5000 was obtained from the Bureau of Animal Industry.

3. Red blood cells: All red blood cells were obtained by cardiac puncture of healthy chickens. The blood was citrated and centrifuged, the sedimented cells were washed three times with normal saline. The packed cells were stored in the refrigerator and a two per cent saline suspension was made fresh each day. No cells were used which showed hemolysis nor which had been stored over three days.

The suitability of all red cells was determined by the hemagglutination test with a previously titered virus before being used.

4. Hemagglutination test procedure: Chemically clean agglutination tubes of approximately 10 mm. diameter were placed in racks with coarse mesh wire bottoms so that the bottoms of the tubes were clearly visible. A modification of the method recommended by the Bureau of Animal Industry was used as shown in table II.

The doubling dilutions of virus were made ranging from 1:5 to 1:1280, shaken and allowed to stand ten minutes. The two per cent suspension of

washed red cells were added to each tube, and the rack shaken well and allowed to stand at room temperature (70° to 75° F.) for 25 to 30 minutes before reading.

TABLE II

Scheme of Hemagglutination Serological Titration

Materials	Tube 1	Tube 2	Tubes 3 to 10	Tube 11
Formalized Newcastle virus	undiluted 0.25 ml.	0.25 ml. of 1:5 dilution	0.25 ml. of progressing twofold dilutions	None
0.85% NaCl solution	0.25 ml.	0.25 ml.	0.25 ml.	0.5 ml.
2% saline suspension of red blood cells	0.25 ml.	0.25 ml.	0.25 ml.	0.25 ml.

5. Hemagglutination-inhibition test procedure: Using positive, negative and suspect serums, the tests were set up and read exactly as in the case of the hemagglutination test, except that the serum to be tested was diluted 1:5 in normal saline and substituted for the plain saline in the hemagglutination test.

The egg yolk extract was prepared by mixing approximately one milliliter of yolk with six milliliters of a normal saline solution, to this was added one milliliter of reagent grade ether and two milliliters of ethylene dichloride and the tube thoroughly shaken. This material was held at 37° C. for 14-16 hours at which time it was centrifuged, the supernatant fluid was drawn off and substituted directly for the 1:5 dilution of serum in the hemagglutination-inhibition test, as above.

All readings and recordings were made by the method of Beach (1948) and Salk (1944) as previously outlined.

Hemagglutination-inhibition titers were determined by dividing the significant figure of the highest dilution of virus giving hemagglutination by the significant figure of the lowest dilution of virus of the hemagglutination-inhibition series showing inhibition. This figure multiplied by five, to compensate for the original 1:5 dilution, represents the final hemagglutination-inhibition titer or value.

B. Experimental Procedures and Results

Experiment I

This experiment consisted of the standardization of the Newcastle virus (Oklahoma strain #7) which was used in the subsequent experimental work. Table III shows the results of an early trial to determine a minimum infective embryo inoculum or minimum lethal dose ("m.l.d."). From this evidence it was indicated that such a value could be obtained. All "m.l.d." determinations were made on eggs from hens with a negative hemagglutination-inhibition titer. In order to eliminate the possibility of "diluting out" the virus, controls were carried on eggs from immune hens as determined by a positive serum HI test. All inoculations were made in the allantoic fluid of 16 day embryos and results were read after 36 hours at 37° C. The results shown in table III led to further experimentation to more accurately determine the "m.l.d." as shown in table IV. The "m.l.d." was found to be 0.01 ml. of a 1:110 dilution of the virus. This was standardized to contain one "m.l.d." per 0.01 ml. of a 1:100 dilution.

TABLE III

Determination of Approximate Range of a Minimum Infective Embryo Inoculum

Inoculum	Dilution of virus							
	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
0.01 ml.	D	D	A	A	A	A	A	A
	D	D	D	A	A	A	A	A
	D	D	D	A	A	A	A	A
0.02 ml.	D	D	D	A	A	A	C	A
	D	D	D	A	A	A	A	A
	D	D	D	A	A	A	A	A
0.04 ml.	D	D	D	A	A	C	A	A
	D	D	D	D	A	A	A	A
	D	D	D	A	A	A	A	A

A - alive; D - dead; C - contaminated.
All eggs were from immune hens as determined by serum HI.

TABLE IV

Determination of "m.l.d." of Newcastle Virus on Susceptible 16-18 day Embryos as Compared to the Resistance of "immune" Embryos

Inoculum	Dilution of virus													
	1:80		1:90		1:100		1:110		1:120		1:130		1:150	
	susc	imm	susc	imm	susc	imm	susc	imm	susc	imm	susc	imm	susc	imm
0.01 ml.	D	A	D	A	A	A	A	A	A	A	A	A	A	A
	D	A	D	A	D	A	A	A	C	A	A	A	C	A
	D	A	D	A	D	A	A	A	C	A	A	A	A	D
	D	A	D	A	D	A	A	A	A	A	A	A	C	A
0.02 ml.	D	A	D	A	D	A	A	A	A	D	A	A	A	A
	D	A	D	A	D	A	A	A	A	A	A	C	A	A
	C	A	D	A	D	A	D	A	A	A	A	A	A	A
	D	A	D	A	D	A	A	A	D	A	A	A	A	A
0.04 ml.	D	A	D	C	D	A	A	A	A	A	A	A	C	A
	D	C	D	A	D	A	A	A	A	A	D	A	A	A
	D	A	D	A	D	A	A	A	A	A	A	A	A	A
	D	A	D	A	D	A	D	A	A	A	A	A	A	A

susc - susceptible (from hens with negative serum HI).
imm - immune (from hens with positive serum HI).
A - alive; D - dead; C - contaminated.

TABLE V

Determination of the Relative Resistance of Both Immune and Susceptible 16 day Embryos to Minimum Infective Inoculations

Status of immunity of the hens.	Inoculum					
	0.8 m.l.d.		2.0 m.l.d.		10.0 m.l.d.	
	alive	dead	alive	dead	alive	dead
"Immune embryos	12	0	11	1	10	2
Susceptible embryos	12	0	2	10	0	12

Table V shows the results of the use of 0.8, 2.0, and 10.0 "m.l.d." on embryos both from hens known to be immune and known to be susceptible. This indicated that an embryo (16-18 days old) from immune hens had enough immunity for protection against 10 "m.l.d." and that embryos from either immune or non-immune hens was not infected with the 0.8 "m.l.d."

Experiment II

This experiment was to determine how closely the hemagglutination-inhibition tests on the blood serum and the yolk of the egg were correlated, also to compare the resistance of the embryo to the titer of the Newcastle antibody found in the fresh egg.

Part A: Two flocks of mated laying hens were available to compare the results of the yolk HI and the resistance of the 16 day embryo to infective doses. Blood serum HI titers were determined on one of the flocks for additional comparative figures, it was impossible to obtain blood samples of the other flock.

Flock #1 consisted of 27 hens representing several breeds, these hens had been vaccinated with live Newcastle virus 37 days before the determinations were made. All three of the above tests were made on this flock of birds.

Flock #2 consisted of 18 white leghorn hens with no history of Newcastle disease and no vaccination had been made. Blood samples were not available from these birds, the determinations were made only on the yolk HI and the embryo resistance tests.

The results of the tests on these two flocks are shown in table VI.

Part B: A comparison between the resulting titers of the blood serum HI and the yolk HI are shown in table VII. These determinations were made on various numbers of birds from different flocks as the opportunity was provided.

The trends shown from these results indicates a lower HI titer was found in the egg yolk than in the blood serum. All of the birds were believed to be carrying some immunity, either from past outbreaks or from recent vaccinations.

TABLE VI

Comparison of Blood Serum and Egg Yolk Hemagglutination-inhibition Titers and the Embryo Resistance of Two Flocks of Chickens.

Flock No.	Hen No.	Blood Serum HI											Egg Yolk HI										Resistance embryo m.l.d.		
		1:1	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	Control	Titer	1:1	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	Control	Titer	0.8	2.0	10.0
1	1	+	−	−	−	−	−	−	−	−	−	640	+	+	+	+	−	−	−	−	−	−	160	A	A	A
	2	+	+	−	−	−	−	−	−	−	−	320	+	+	+	−	−	−	−	−	−	−	160	A	I	A
	3	+	+	−	−	−	−	−	−	−	−	320	+	+	+	+	−	−	−	−	−	−	80	A	A	A
	4	+	+	+	−	−	−	−	−	−	−	160	+	+	+	+	−	−	−	−	−	−	80	A	A	D
	5	+	−	−	−	−	−	−	−	−	−	640	+	+	−	−	−	−	−	−	−	−	320	A	A	A
	6	+	+	−	−	−	−	−	−	−	−	320	+	+	+	−	−	−	−	−	−	−	160	A	A	I
	7	+	+	−	−	−	−	−	−	−	−	320	+	+	+	+	−	−	−	−	−	−	80	A	A	A
	8	+	+	+	+	+	+	+	−	−	−	10	+	+	+	+	+	+	+	+	−	−	5	A	D	D
	9	+	−	−	−	−	−	−	−	−	−	640	+	+	+	−	−	−	−	−	−	−	160	A	A	A
	10	+	+	−	−	−	−	−	−	−	−	320	+	+	+	−	−	−	−	−	−	−	160	A	A	A
	11	+	+	−	−	−	−	−	−	−	−	320	+	+	+	+	−	−	−	−	−	−	80	A	I	A
	12	+	−	−	−	−	−	−	−	−	−	640	+	+	−	−	−	−	−	−	−	−	320	A	A	A
	13	+	+	+	−	−	−	−	−	−	−	160	+	+	+	−	−	−	−	−	−	−	160	A	A	D
	14	+	+	−	−	−	−	−	−	−	−	320	+	+	+	+	−	−	−	−	−	−	80	I	A	A
	15	+	+	−	−	−	−	−	−	−	−	320	+	+	+	−	−	−	−	−	−	−	160	A	A	A
	16	+	−	−	−	−	−	−	−	−	−	640	+	+	+	+	−	−	−	−	−	−	80	A	A	I
	17	+	+	+	−	−	−	−	−	−	−	160	+	+	+	+	−	−	−	−	−	−	80	A	A	D
	18	+	+	+	−	−	−	−	−	−	−	160	+	+	+	+	−	−	−	−	−	−	80	A	A	A
	19	+	+	−	−	−	−	−	−	−	−	320	+	+	+	+	−	−	−	−	−	−	160	A	I	A
	20	+	+	+	+	−	−	−	−	−	−	80	+	+	+	+	+	+	+	+	−	−	5	A	D	I
	21	+	+	−	−	−	−	−	−	−	−	320	+	+	+	+	−	−	−	−	−	−	80	A	A	A
	22	+	+	+	−	−	−	−	−	−	−	160	+	+	+	−	−	−	−	−	−	−	160	A	A	A
	23	+	+	+	−	−	−	−	−	−	−	160	+	+	+	+	−	−	−	−	−	−	80	A	A	I
	24	+	−	−	−	−	−	−	−	−	−	640	+	+	−	−	−	−	−	−	−	−	320	A	A	A
	25	+	+	−	−	−	−	−	−	−	−	320	+	+	+	+	−	−	−	−	−	−	80	A	A	A
	26	+	−	−	−	−	−	−	−	−	−	640	+	+	+	−	−	−	−	−	−	−	160	I	I	I
	27	+	+	+	+	−	−	−	−	−	−	80	+	+	+	+	−	−	−	−	−	−	80	A	C	C
2	1												+	+	+	+	+	+	+	−	−	−	10	A	D	D
	2												+	+	+	+	+	+	+	+	−	−	5	A	D	D
	3												+	+	+	+	+	+	−	−	−	−	20	D	D	A
	4												+	+	+	+	+	+	+	+	−	−	5	I	D	D
	5												+	+	+	+	+	+	+	+	−	−	5	A	D	D
	6												+	+	+	+	+	+	+	+	−	−	5	A	D	D
	7												+	+	+	+	+	+	−	−	−	−	20	D	I	I
	8												+	+	+	+	+	+	+	+	−	−	5	A	D	D
	9												+	+	+	+	+	+	+	−	−	−	10	A	D	D
	10												+	+	+	+	+	+	+	±	−	−	10	D	A	D
	11												+	+	+	+	+	+	+	−	−	−	5-10	A	I	D
	12												+	+	+	+	+	+	+	−	−	−	10	A	D	D
	13												+	+	+	+	+	+	+	−	−	−	10	A	D	I
	14												+	+	+	+	+	+	+	−	−	−	10	A	D	I
	15												+	+	+	+	+	+	+	+	−	−	5	D	D	C
	16												+	+	+	+	+	+	−	−	−	−	20	A	I	D
	17												+	+	+	+	+	+	−	−	−	−	20	D	D	D
	18												+	+	+	+	+	+	+	+	−	−	5	D	D	D

A - alive; D - dead; C - contaminated; I - infertile.

From the data shown in table VII, it is seen that in 51 out of 56 diagnoses, the results were the same by both methods. Three birds gave negative results by the yolk HI test as compared to positive results by the serum HI method. In no case was a "false positive" shown by the yolk HI in contrast to a negative blood titer.

TABLE VII

Comparison of Blood Serum and Egg Yolk HI Titers.

Group No.	Hen No.	HI titer			
		Blood	Diag.	Yolk	Diag.
A	1	160	Pos.	80	Pos.
	2	160	Pos.	160	Pos.
	3	80	Pos.	80	Pos.
	4	320	Pos.	320	Pos.
	5	80	Pos.	40	Neg.
	6	160	Pos.	80	Pos.
	7	160	Pos.	160	Pos.
	8	320	Pos.	160	Pos.
	9	640	Pos.	320	Pos.
	10	160	Pos.	160	Pos.
	11	320	Pos.	80	Pos.
	12	160	Pos.	160	Pos.
	13	160	Pos.	80	Pos.
	14	320	Pos.	80	Pos.
	15	640	Pos.	160	Pos.
	16	320	Pos.	160	Pos.
	17	80	Pos.	80	Pos.
	18	320	Pos.	160	Pos.
	19	160	Pos.	80	Pos.
	20	160	Pos.	80	Pos.
	21	320	Pos.	320	Pos.
	22	80	Pos.	160	Pos.
	23	320	Pos.	Male	-
B	1	20	Neg.	40	Neg.
	2	160	Pos.	80	Pos.
	3	320	Pos.	160	Pos.
	4	80	Pos.	160	Pos.
	5	160	Pos.	80	Pos.
	6	160	Pos.	320	Pos.
	7	80	Pos.	80	Pos.
	8	640	Pos.	160	Pos.
	9	160	Pos.	160	Pos.
	10	160	Pos.	80	Pos.
	11	160	Pos.	80	Pos.
	12	160	Pos.	160	Pos.
	13	320	Pos.	160	Pos.
C	1	40	Neg.	20	Neg.
	2	160	Pos.	80	Pos.
	3	80	Pos.	40	Neg.
	4	320	Pos.	160	Pos.
	5	320	Pos.	160	Pos.
	6	5	Neg.	-	-*
	7	80	Pos.	80	Pos.
	8	160	Pos.	80	Pos.
	9	160	Pos.	160	Pos.
D	1	320	Pos.	160	Pos.
	2	160	Pos.	160	Pos.
	3	160	Pos.	80	Pos.
	4	320	Pos.	5	Neg.
	5	160	Pos.	80	Pos.
	6	320	Pos.	160	Pos.
	7	80	Pos.	10	Neg.
	8	160	Pos.	80	Pos.
	9	160	Pos.	320	Pos.
	10	80	Pos.	80	Pos.
	11	160	Pos.	160	Pos.
	12	320	Pos.	40	Neg.

* - This hen had suddenly dropped in egg production and was observed to have a respiratory trouble resembling Newcastle disease.

Experiment III

This experiment was conducted in cooperation with the Poultry Department, Oklahoma A. & M. College, Stillwater, Oklahoma.

Following a respiratory outbreak accompanied by greatly reduced egg production among some hens in the egg production contest pens, the immune status of some of the birds in representative pens was determined. Table VIII gives a graphic picture of the course of the outbreak. The suspicion of Newcastle disease increased when it was observed that no pen with birds vaccinated with live virus and only two pens vaccinated with modified virus suffered any noticeable symptoms.

The results of this experiment are shown in table IX. Determinations were made on the hemagglutination-inhibition titer of the egg yolk and compared to the resistance of 16 day embryos when challenged with 0.8, 2.0, and 10.0 "m.l.d." of Newcastle virus. Since these birds were contestants in an egg laying contest, it was not practical nor advisable to obtain any blood samples.

The birds in this test did not have any history of having had Newcastle disease as far as could be ascertained. The history of the vaccination of the different pens is indicated in table VIII. The length of time since these vaccinations had been made was not available in every case. Pen #11 had been vaccinated with a live virus vaccine only a few weeks before they were placed in the contest. Apparently the outbreak began in the pens adjacent to this most recently vaccinated pen, whether or not these birds in pen #11 were still affected with live virus is not known. Other pens vaccinated with the live virus vaccine had been vaccinated for longer periods of time before they were placed in the contest.

TABLE VIII

Occurrence of a Respiratory Outbreak Resembling Newcastle Disease Among Some Contest Pens, Oklahoma A. & M. College, Stillwater, Oklahoma.

Pen No.	Vaccination	Oct. 1948 Weeks 1	2	3	4	Nov. 1948 Weeks 1	2	3	4	Dec. 1948 Weeks 1	2	3	4	Jan. 1949 Weeks 1	2	3	4	Feb. 1949 Weeks 1	2	3	4
1				■																	
2				■																	
3	live virus				■																
4					■																
5	live virus														■						
6	dead virus				■										■						
7					■														■		
8			■																■		
9			■																		
10			■																		
11	live virus												■								
12													■								
13														■							
14							■							■							
15							■								■						
16												■			■						
17											■	■									
18											■		■								
19													■								
20	mod. live				■																
21	live virus								■												
22									■												
23	mod. live						■														
24							■														
25	live virus																				
26	mod. live																				
27	mod. live						■														
28	mod. live						■														
29							■														
30							■														
31							■														
32	live virus																				
33	live virus					■															
34						■															
35						■															
36							■														
37	mod. live																				
38				■																	
39	live virus																				
40	mod. live						■														
47							■														
48						■															

Note: This chart does not represent all of the contest pens.

TABLE IX

Determination of Antibody Level in Egg Yolk by HI Test Compared to the Resistance of 15-17 day Embryos from Chickens Suspected of Newcastle.

Pen No.	Hen No.	Hemagglutination-inhibition test Virus dilutions 1/1	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	C	HI Titer	Resistance of Embryo to Virus "m.l.d." 0.8	2.0	10.0	Immune Status
	2	+	-	-	-	-	-	-	-	-	-	320	A	A	A	Pos.
	3	+	-	-	-	-	-	-	-	-	-	320	A	A	I	Pos.
	4	+	-	-	-	-	-	-	-	-	-	320	A	A	I	Pos.
	5	-	-	-	-	-	-	-	-	-	-	640	A	A	A	Pos.
6	6	+	-	-	-	-	-	-	-	-	-	320	A	A	A	Pos.
	8	+	+	+	+	+	+	+	-	-	-	5	A	D	D	Neg.
	9	+	-	-	-	-	-	-	-	-	-	320	A	A	A	Pos.
	C	+	-	-	-	-	-	-	-	-	-	320	A	A	A	Pos.
	C	+	-	-	-	-	-	-	-	-	-	320				Pos.
	0	+	-	-	-	-	-	-	-	-	-	320	A	A	A	Pos.
	3	+	+	+	+	+	+	+	-	-	-	5	A	D	D	Neg.
	5	+	+	-	-	-	-	-	-	-	-	160	A	A	D	Pos.
	9	+	-	-	-	-	-	-	-	-	-	320	A	A	A	Pos.
12	9	+	-	-	-	-	-	-	-	-	-	320				Pos.
	A	+	+	+	+	+	-	-	-	-	-	20	I	A	I	Susp.
	A	+	+	+	+	+	+	+	-	-	-	10				Neg.
	B	+	+	+	+	+	+	+	-	-	-	5	D	D	D	Neg.
	B	+	+	+	+	+	+	+	-	-	-	5				Neg.
	6	+	-	-	-	-	-	-	-	-	-	320	I	A	A	Pos.
	6	+	-	-	-	-	-	-	-	-	-	320				Pos.
13	8	+	-	-	-	-	-	-	-	-	-	160	A	A	A	Pos.
	8	+	+	-	-	-	-	-	-	-	-	320	A	A	I	Pos.
	C	+	-	-	-	-	-	-	-	-	-	320	A	D	A	Pos.
	0	+	-	-	-	-	-	-	-	-	-	320	A	A	A	Pos.
	0	+	+	-	-	-	-	-	-	-	-	160				Pos.
	5	+	+	+	+	+	+	+	-	-	-	5	D	D	I	Neg.
14	7	+	-	-	-	-	-	-	-	-	-	320	A	A	I	Pos.
	9	+	-	-	-	-	-	-	-	-	-	320	C	A	I	Pos.
	B	+	+	-	-	-	-	-	-	-	-	160	A	A	D	Pos.
	C	+	-	-	-	-	-	-	-	-	-	320	A	A	A	Pos.
15	B	+	-	-	-	-	-	-	-	-	-	320	A	A	A	Pos.

A - alive; D - dead; C - contaminated; I - infertile.

TABLE IX (cont'd)

Pen No.	Hen No.	Hemagglutination-inhibition test: Virus dilutions 1/1	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	C	HI Titer	Resistance of Embryo to Virus "m.l.d." 0.8	2.0	10.0	Immune Status
	1	+	-	-	-	-	-	-	-	-	-	320	A	A	D	Pos.
	2	+	-	-	-	-	-	-	-	-	-	320	A	A	A	Pos.
17	3	+	+	-	-	-	-	-	-	-	-	160	A	A	A	Pos.
	B	+	-	-	-	-	-	-	-	-	-	320	I	A	I	Pos.
	C	+	-	-	-	-	-	-	-	-	-	320	A	I	I	Pos.
	1	+	-	-	-	-	-	-	-	-	-	320	A	A	A	Pos.
	2	+	+	+	+	+	+	+	+	+	-		A	D	D	Neg.
18	3	+	-	-	-	-	-	-	-	-	-	320	A	A	D	Pos.
	5	+	-	-	-	-	-	-	-	-	-	320	I	A	I	Pos.
	9	+	+	+	+	+	+	+	-	-	-	5	I	I	I	Neg.
	1	+	-	-	-	-	-	-	-	-	-	320	A	A	I	Pos.
	2	+	+	+	+	+	+	+	-	-	-	5	I	D	I	Neg.
	3	+	-	-	-	-	-	-	-	-	-	320	A	C	A	Pos.
	4	+	-	-	-	-	-	-	-	-	-	320	A	A	A	Pos.
24	5	+	-	-	-	-	-	-	-	-	-	320	A	A	D	Pos.
	6	+	-	-	-	-	-	-	-	-	-	320	A	A	D	Pos.
	9	+	-	-	-	-	-	-	-	-	-	320	A	A	I	Pos.
	0	+	+	+	+	+	+	+	-	-	-	5	D	D	I	Neg.
	C	+	-	-	-	-	-	-	-	-	-	320	A	A	I	Pos.
	0	+	-	-	-	-	-	-	-	-	-	320	D	A	C	Pos.
	2	+	-	-	-	-	-	-	-	-	-	320	A	D	I	Pos.
29	6	+	+	+	+	+	+	+	-	-	-	5	I	I	I	Neg.
	8	+	+	-	-	-	-	-	-	-	-	160	A	A	D	Pos.
	A	+	-	-	-	-	-	-	-	-	-	320	I	A	A	Pos.
	1	+	-	-	-	-	-	-	-	-	-	320	A	A	A	Pos.
	1	+	-	-	-	-	-	-	-	-	-	320				Pos.
	5	+	-	-	-	-	-	-	-	-	-	320	A	A	A	Pos.
30	8	+	+	-	-	-	-	-	-	-	-	160	A	A	D	Pos.
	9	+	+	-	-	-	-	-	-	-	-	160	I	I	I	Pos.
	B	+	-	-	-	-	-	-	-	-	-	320	I	A	I	Pos.
31	0												I	I	I	-
	5	+	-	-	-	-	-	-	-	-	-	320	A	A	I	Pos.
	0	+	+	-	-	-	-	-	-	-	-	160	A	A	D	Pos.
	4	+	+	+	+	+	-	-	-	-	-	20-80	I	D	I	Neg.
35	8	+	+	+	+	+	+	+	-	-	-	5	A	D	D	Neg.
	B	+	-	-	-	-	-	-	-	-	-	320	A	A	A	Pos.

A - alive; D - dead; C - contaminated; I - infertile.

TABLE IX (cont'd)

Pen No.	Hen No.	Hemagglutination-inhibition test — Virus dilutions										HI Titer	Resistance of Embryo to Virus "m.l.d."			Immune Status
		1/1	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	C		0.8	2.0	10.0	
36	1	+	+	+	+	+	+	+	−	−	−	5	D	D	I	Neg.
	2	−	−	−	−	−	−	−	−	−	−	640	A	A	A	Pos.
	4	+	+	−	−	−	−	−	−	−	−	160	A	I	I	Pos.
	B	+	+	±	±	±	−	−	−	−	−	20-80	A	D	I	Neg.
47	1	+	+	+	+	+	+	+	−	−	−	5	D	D	D	Neg.
	2	+	−	−	−	−	−	−	−	−	−	320	A	A	A	Pos.
	4	+	−	−	−	−	−	−	−	−	−	320	I	I	I	Pos.
	C	+	+	+	+	+	+	+	−	−	−	5	A	D	D	Neg.
48	0	+	+	+	+	+	+	+	−	−	−	5	D	D	D	Neg.
	3	+	+	+	+	−	−	−	−	−	−	40	I	I	I	Neg.
	4	+	−	−	−	−	−	−	−	−	−	320	A	A	I	Pos.
	5	+	+	+	+	+	+	+	−	−	−	5	D	D	D	Neg.
	8	+	−	−	−	−	−	−	−	−	−	320	A	A	I	Pos.
	9												I	I	I	-
	A	+	+	+	+	−	−	−	−	−	−	40	A	D	D	Neg.
	C	+	+	+	+	+	+	+	−	−	−	5	I	D	I	Neg.
A	1	..Control egg..											A	D	D	
	2	..Control egg..											A	D	D	
	3	..Control egg..											A	A	D	
	4	..Control egg..											A	D	I	
	5	..Control egg..											A	A	D	
	6	..Control egg..											A	D	D	
B	7	..Control egg..											A	D	D	
	8	..Control egg..											A	D	D	
	9	..Control egg..											A	A	D	
	10	..Control egg..											A	A	I	
	11	..Control egg..											A	A	D	
	12	..Control egg..											A	D	D	
	13	..Control egg..											A	D	D	
	14	..Control egg..											D	D	D	
	15	..Control egg..											A	D	D	
	16	..Control egg..											I	D	I	
	17	..Control egg..											A	A	D	
	18	..Control egg..											A	I	I	

A - alive; D - dead; C - contaminated; I - infertile.

Virus titer - 1:320

DISCUSSION

The results of Experiment I indicate the same results regarding the immunity of the embryo as found by Brandly, et al. (1946). This transfer of the immune substances from the yolk to the tissues of the embryo is significant from the standpoint of virus culture work and in providing a better understanding of the congenital immunity conferred to the chick. The relatively high titer of the newly hatched chicks from immune hens, although passive in nature and consequently of short duration, undoubtedly plays a very important part in attempts to develop an active immunity in these chicks during the early weeks of life.

The relative values of the blood serum and yolk HI titers and the resistance of the embryo in determining the diagnostic titers are brought out in part by Experiment II. The correlation of the results by each of the methods indicates the yolk HI test to correspond favorably with the blood serum HI titer. It is interesting to note that in no case was a positive reading of the yolk HI found to occur in eggs from a hen read as negative by the serum determination. This might indicate, in the case of a negative egg from a positive hen, that some hens carrying protective antibody do not transmit this characteristic to their offsprings. Outbreaks in chicks less than three weeks old from supposedly immune hens would seem to add evidence to this possibility. The presence of non-specific agglutinative inhibitors or receptor destroying enzymes (RDE) or even the presence of live virus in the yolk of such eggs could account for the negative results from these positive hens. Every effort was made in these yolk determinations to exclude any albumen from the sample taken, the presence of which would inhibit the reaction as shown by Lamni and Beard (1948).

Although not reported in the present paper, some efforts have been made by the author to determine which fraction of the yolk carries the immune properties. Apparently the globulins, which are soluble in saline are partially released from the lecithoprotein livetin by the fat extraction with the ether and by removal of the lipoids, such as lecithin, by their solubility in the ethylene dichloride. The saline-soluble beta and gamma globulins have been partially separated by successive isoelectric precipitations. As far as can be determined now, both fractions demonstrate some immune substances.

In Experiment III, the yolk HI and the resistance of the embryo to the Newcastle virus were found to compare very closely. Partially on the basis of these results, it can be assumed that the respiratory outbreak was Newcastle disease.

An interesting point to note is that the outbreak began in pens #9 and #10 which are adjacent to the pen #11 which was vaccinated with live virus vaccine just a few weeks before the birds were placed in the contest. This spread from an affected pen to the adjacent pens was observed throughout the outbreak.

There appears to be a definite trend in the number of birds found with a low titer as compared to the length of time since that pen was affected. Of the pens in which the immune status was determined, pens #35 and #48 were among the earliest to show symptoms of the disease, in both of these pens a large percentage of the birds were found to transmit little or no antibody to the egg. Pens #6 and #13 were among the last to be affected during the outbreak and also show a more nearly complete immunity. These correlations would indicate that the immunity developed by the natural outbreak may diminish rapidly and apparently could not be expected to protect the birds

or newly hatched chicks from these hens very long after hatching. The protection apparently afforded pen #6 by a dead virus vaccine in the early part of the outbreak diminished sufficiently in the four month interval to render them susceptible. The outbreaks in the pens vaccinated with modified virus would also probably show that the time interval since vaccination had been too long to provide protection. The escape of many pens which had been vaccinated with modified virus or dead virus and the failure of any pen which had not been vaccinated to escape the disease is significant.

It is impossible to determine the blood serum HI titer of these birds, but it might prove interesting to have this additional information.

It is planned at some later date to follow the immunity pattern of a group of birds by testing at intervals before vaccination and several times after vaccination to determine how rapidly the titer falls below the protective level.

SUMMARY

The history and development of the hemagglutination test and its application to the diagnosis of Newcastle disease are reviewed. The Francis inhibitor and the receptor destroying enzymes and their effect upon the hemagglutination tests are briefly reported.

An Oklahoma virus (strain #7) has been isolated and identified. It was determined that an "immune" 16 day embryo could resist infection with ten or more "m.l.d." of virus.

A number of birds were tested by the blood serum HI, yolk HI and by the embryo resistance procedures. The results were found to compare favorably, although the yolk titer was somewhat lower than the blood serum titer.

A respiratory outbreak among some egg production contest birds resembling Newcastle disease was observed. The yolk HI titer and embryo resistance values were determined for some representative birds from these pens. It was found that a large percentage of the birds did carry an immunity. Some correlation was observed in the trend of the number of immune birds in comparison to the time elapsed since a high degree of immunity could be expected. Also, observations were made upon the probable degree of immunity conferred by the different methods of vaccination. In this respect, live virus vaccine apparently provided the longest protective immunity, closely approaching the immunity following a natural outbreak. It appears that the immune status may fall below a protective value before a year has elapsed.

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