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THE EFFECT OF BETA-PROPIOLACTONE ON SOME OF THE INFECTIOUS AGENTS THAT PRODUCE DISEASES OF CHICKENS

THESIS FOR THE DEGREE OF PH. D.
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ACHIT CHOTISEN

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THESIS

This is to certify that the

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The Effect of Beta-propiolactone
on Some of the Infectious Agents that
Produce Diseases of Chickens.

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Achit Chotisen

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THESIS

THE EFFECT OF BETA-PROPIOLACTONE ON SOME OF THE INFECTIOUS AGENTS THAT PRODUCE DISEASES OF CHICKENS

by

Achit Chotisen

A THESIS

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Beta-propiolactone in 0.025 per cent concentration in Newcastle disease infected allantoic fluid destroyed infectivity without destroying antigenicity of the virus. A vaccine against Newcastle disease, using this concentration of beta-propiolactone, prevented infection in experimental chickens. Of 43 vaccinated birds, none showed symptoms of Newcastle disease after being challenged with a virulent strain of Newcastle disease virus 16 days following vaccination. In a group of 39 non-vaccinated birds, 38 birds (97.4%) developed symptoms or died from the challenging dose of virus. One bird in this group remained well. Serological tests on paired sera showed increases in both hemagglutination inhibition and neutralizing antibodies in the vaccinated group. In contrast, the non-vaccinated group did not demonstrate an increase in antibodies. A concentration of 0.025 per cent beta-propiolactone also destroyed the infectivity of infectious bronchitis and infectious laryngotracheitis virus contained in allantoic fluid but not completely *Salmonella pullorum*. The results suggest the possibility of making a multiple virus vaccine for prevention of these diseases in chickens. In 0.1 per cent concentration of beta-propiolactone, *Salmonella pullorum* was killed but the antigenicity of Newcastle disease virus was destroyed. All chickens vaccinated with 0.1 per cent beta-propiolactone in Newcastle disease virus allantoic fluid were susceptible to infection

by challenging with Newcastle disease virus. This type of inactivated vaccine is preferable to live virus vaccine because of its high antigenicity and ability to destroy infectivity of the virus.

To

LUANG VIPACHANA SUTHAPOCHANA
(Arch Chotisen)

and

NANG VIPACHANA SUTHAPOCHANA
(Huang Chotisen)

My beloved father and mother

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I. INTRODUCTION

Beta-propiolactone (B.P.L.) was found to be an effective virucide and of low toxicity by Mangun *et al*. (1951). Hartman *et al*. (1951) also reported that a number of viruses as well as bacteria were killed by the action of B.P.L. Beta-propiolactone mixed with Newcastle disease virus suspension held at 37°C for 1 to 2 hours effectively destroyed the virus.

Newcastle disease is an infectious, highly contagious and destructive virus infection of poultry and wild birds. Certain mammals, including man, have been reported to be susceptible to this disease. Newcastle disease is caused by a virus which resembles in some respects the viruses of influenza and mumps.

Newcastle disease in poultry causes serious economic losses. High mortality rates among young chickens are reported throughout the world (Beaudette, 1943, 1948, 1951). Chicks that survive an outbreak are sometimes impaired with respect to growth and efficiency of feed utilization. The decrease in egg production after an attack of the disease, together with the serious damage to egg quality is of sufficient magnitude to be of economic importance (Berg *et al*., 1947; Knox, 1950).

The object of this study was to produce a satisfactory vaccine against Newcastle disease, with the following qualities:

(1) Incapable of producing the disease in susceptible animals; (2) Capable of conferring protection against Newcastle disease for an adequate period of time; (3) Non toxic; (4) Free of other infectious agents.

II. HISTORY

A. Newcastle Disease

Newcastle disease (NCD) was first known as a highly fatal infection of poultry prevalent in the Dutch East Indies. The disease was first reported by Kraneveld in 1926 (Beaudette, 1943). Kraneveld demonstrated that the causative agent was present in the secretion from the respiratory tract. He eliminated the possibility that the cause was bacterial in nature by Berkefeld filtration. The disease was also reported to cause an outbreak in poultry near Newcastle on the Tyne in England by Doyle (1927). Doyle demonstrated that the cause is a filtrable virus and is present in the body fluids, organs and excretions of affected birds. The disease resembles fowl plague, but it can be differentiated by serological tests. Doyle found that there is no cross immunity between NCD and fowl plague and that each virus may be completely neutralized by the homologous immune serum. Doyle proposed the name Newcastle disease in describing the disease.

During the decade following its recognition, Newcastle disease was reported from India, the Philippine Islands, Korea, Japan, Australia, Ceylon, Kenya, Palestine and Syria; in fact, the disease is widely spread throughout the world (Beaudette, 1943, 1948, 1951). In the initial appearances of the malady, the disease was seldom recognized as NCD, therefore, it was

given many names, such as pseudo-fowl pest, pseudovogelpest, avian pest, avian distemper, Ranikhet, Madras fowl pest, Chosen disease and so forth (Brandly *et al.*, 1946 a). The similarity in symptoms of these diseases reported from the orient and England brought about an exchange of viruses for comparison. It was found that regardless of name and minor differences in symptoms, the viruses from the Dutch East Indies, India, Philippine Islands and England were immunologically identical. Later, it was shown by Japanese workers that their viruses were identical with the Philippine virus (Brandly *et al.*, 1946 a). Thereafter most of the various names under which the disease had been known were discontinued and NCD has been recognized as the proper name of this disease.

In the United States this disease was described in California by Beach in 1941 as a nervous disorder occurring after or towards the end of outbreaks of a disease which was thought to be chicken bronchitis. He also suggested that the disease might be due to a virus or to some dietary disturbances. Later Stover (1942 a, b) described this disease as "a respiratory nervous disorder" of chickens that could be transmitted to other chickens by contact. The filtrability of the infective material was also demonstrated, and, by cross immunity experiment, it was shown that the virus of the respiratory disorder was distinct from the virus of infectious bronchitis and from infectious laryngotracheitis. Beach (1942) proposed the name "Avian Pneumoencephalitis" for this disease. The unusually

mild character and low mortality rate which characterized the malady upon its initial appearance in America allayed suspicion that it might be NCD. Later Beach (1944 b) observed the highly virulent nature of the cultured pneumoencephalitis virus and suspected that it might be related to the virus of NCD. Small amounts of anti Newcastle and anti pneumoencephalitis sera were used for *in vitro* neutralization of pneumoencephalitis virus. The results indicated that pneumoencephalitis virus was immunologically identical with Newcastle disease virus (NDV).

Burnet and Ferry (1934) found that NDV is easily propagated in embryonating chicken eggs and reaches high titer (10^8 to 10^9 infectious units per ml.) in the chorioallantoic fluid. By ultrafiltration experiments they estimated the size of NDV particles to be 80-120 mu.

The virus can pass through Berkefeld V, N, W, Chamberland candles L_3 and L_5 and asbestos discs (Doyle, 1927; Stover, 1942 a, b; Beaudette, 1943). Bang (1946, 1947) observed the shape of NDV by electron microscope and found it to be spherical in shape in allantoic fluid, but this form could be converted into filamentous forms if the virus was concentrated by ultracentrifugation and suspended in water. Cunha *et al*. (1947) concentrated NDV from chorioallantoic fluid and described it as sperm shaped. The head measured 70 x 180 mu. and tail 500 mu. long.

NDV produces disease in chickens, turkeys, pheasants, guinea fowl, sparrows, crows, francolines and parrots (Beaudette, 1943). Experimental infection has been achieved in chick

embryos, ducks, geese, pigeons and several varieties of wild birds. Mice, hamsters, cotton rats and monkeys may be infected experimentally with NDV, resulting in a meningoencephalitis.

B. Immunization

Earlier efforts to develop a satisfactory vaccine against NCD were by physical means or chemical agents. In the process of attenuation or inactivation of the virus, the results were often irregular and unsatisfactory (Beaudette, 1943). The procedure for attenuation or inactivation of the virus was responsible for inadequate or excessive denaturation of the virus protein. The irregularities in initial virus content of infected tissues added to the variable and unsatisfactory results.

1. Killed Virus Vaccine

Topacio and Coronel (1939) failed to immunize birds with a vaccine made by using infected tissue or "crop virus" treated with chloroform, formalin, lactic acid or merthiolate.

Haddow and Idnani (1941) developed a formalin treated vaccine from infected tissues by adsorption of the virus on aluminium hydroxide gel (adjuvant). The principle involved here was concentration of the virus by the adsorption process followed by inactivation of the virus with dilute formalin. It was presumed that after injection of the vaccine, the adsorbed virus would be gradually released in minimal quantities

into the birds' systems and would thus produce immunity with no apparent symptoms. The vaccinated birds resisted a challenge inoculation of 10 - 100 or more minimum lethal doses of virus between the eighth and the twenty-first day after vaccination.

Iyer and Dobson (1941) prepared a vaccine from infected chicken embryos by suspending the embryos in a buffer solution and treated them with gentian violet. The vaccine was placed at 37°C for 72 hours to complete the inactivation. The results of immunization tests in chickens were irregular and unsatisfactory. Later Iyer (1943) reported that satisfactory results were obtained from this same type of vaccine. At this time he also reported that his tests proved that formalin had no value in the preparation of a suitable vaccine from egg produced virus.

Beach (1942) reported encouraging results obtained in preliminary experiments in immunization of young chickens with formolized saline suspensions of infected chicken embryos. His results showed that a high degree of protection against avian pneumoencephalitis (NCD) could be obtained by this vaccine. The vaccine contained 0.1 or 0.2 per cent formalin. Two doses of vaccine were more effective than a single dose when tested in chickens of more than 7 days of age. Chickens vaccinated at 2 or 3 days of age were inadequately protected by a single or double injection of vaccine.

Later Beach (1944 a) reported that two injections of formolized chicken embryo vaccine yielded a valuable, although incomplete, degree of protection against the natural infection in chickens. The chickens were able to withstand a severe natural outbreak of NCD with relatively low mortality and only a slight decrease in egg production. An effective vaccine could be made with an embryo content varying from 12.5 to 50 per cent. It had to be used from 40 to 509 days after preparation.

Brandly *et al*. (1946 c) reported that formalin inactivated vaccine produced higher protection than ultraviolet irradiation (1600-1800 A°) inactivated vaccine. Vaccines containing 0.25 per cent formalin were found to possess excellent keeping quality. Formalin inactivated vaccines retained their immunogenicity well for a period up to 471 days under a pH range from 5 to 9. The best formalin fixed vaccines were obtained by using high titer virus. The minimal virus titer for maximum vaccine potency had to be 10^7 embryo lethal doses per 0.05 ml. of inoculum from egg material. For vaccine production, eggs produced by NCD immune hens, and containing the yolk sac with content, were inferior to vaccines made from the same eggs but without the yolk sac. Addition of the yolk material from the eggs of susceptible hens did not alter the potency of the vaccine. Formalin inactivated vaccines produced a temporary immunity. Some immunity was present up to 4 months in some cases but in others only slight or negligible refractory was found 3 weeks

after vaccination. The addition of certain adjuvants, such as lanolin, mineral oils and alumina gel into the formolized vaccine increased the duration of resistance without producing unduly severe tissue reaction. When killed *Mycobacterium butyricum* was employed, severe inflammatory reaction and tuberculin sensitivity developed.

Brandly *et al*. (1946 d) reported the presence of specific antiviral activity in the yolk material of eggs laid by the Newcastle disease vaccinated hens. This was true also for eggs from hens recovered from NCD. Embryonic tissues and allantoamnionic membranes also possessed the property of virus neutralization. Vaccines made from eggs that contained this antiviral activity appeared to be inferior to vaccine made from the eggs from susceptible hens. Congenital passive immunity was found for about two weeks after hatching. This immunity interfered with active immune response to vaccination.

Acevedo and Mendoza (1947) reported satisfactory results from chicken embryo tissue vaccine inactivated by 0.05 per cent formalin and crystal violet. Another type of vaccine containing 0.5 per cent potassium alum, 0.7 per cent sodium borate and 0.1 per cent formalin was also found to enhance the antibody production. Later Coronel (1947) announced a successful vaccine using chicken embryo tissue suspended in alum gel and inactivated with 0.1 per cent formalin.

Adler *et al*. (1951) stated that formalinized tissue vaccine did not prevent the spread of the disease as was shown

by isolation of NDV from vaccinated birds exposed to the disease. Vaccination, however, prevented serious loss.

Doll *et al*. (1951) confirmed that commercially killed NCD vaccine did not protect against NCD infection. They described paralytic and fatal infection after challenging exposure to virulent NDV.

2. Live (Attenuated) Virus Vaccine

Immunization of poultry against NCD with a killed virus had met with little success. However, chickens recovered from naturally occurring infection, or challenged with a potent virus developed an immunity which appeared to be permanent. Attenuated strains of NDV have been developed by numerous workers for the production of durable immunity in chickens.

Iyer and Dobson (1940) reported that serial passage of the Hertfordshire strain of Newcastle disease virus on the chorioallantoic membrane of embryonating eggs attenuated the virus. This attenuated virus was satisfactory for a vaccine. From 14 to 33 serial passages were found to be necessary for attenuation of the virus. This cultivated virus did not regain its original virulence by passages through chickens. Inoculation of chickens with this attenuated virus protected them against a challenging dose of 10^8 lethal doses. Later Van Roekel *et al*. (1948) found that an attenuated strain of NDV could immunize chickens when applied by the cutaneous "stick" method in the web of the wing. A general systemic

reaction was noticed in some birds in the form of a slight decrease in activity and appetite. In laying birds, egg production and egg quality were adversely affected, as is the case in a natural outbreak.

Komarov and Goldsmit (1946) reported that after ten intracerebral passages of virulent fowl virus through ducklings, an attenuation of NDV developed. Fowls inoculated with this attenuated NDV did not develop symptoms of NCD and were immune to challenge. Later duck embryo adapted strains of NDV were reported by Markham _et al_. (1949) and Clancy _et al_. (1949). The vaccine was applied by the wing web method. Some of the 4 weeks old birds showed slight depression but continued to feed and grow normally.

Hitchner and Johnson (1948) recommended the use of B 1 strain of NDV, which was of low virulence and could be used to vaccinate chickens of all ages including day-old chicks. Vaccination was accomplished by intranasal instillation of 0.05 ml. of the vaccine. All vaccinated birds were fully protected from the disease when challenged 2 days after vaccination. Doll _et al_. (1950) reported that, as indicated by hemagglutination inhibition test, the immunizing action of the B 1 vaccine was more efficient by intranasal instillation than by intramuscular injection. After vaccination, respiratory symptoms could be seen in birds of all ages. Reduction in egg production, from 20 to 50 per cent for a period of 2 to 4 weeks, and 10 percent mortality in day-old chicks made this type of vaccine unsuitable.

Bankowski and Boynton (1948) found that NDV could be propagated _in vitro_ in modified Simm and Sander's medium containing bovine serum ultra filtrate. The virus propagated by this method seemed to be reduced in virulence when inoculated into chickens. Later Bankowski (1950) confirmed that by 50 serial passages in Simm and Sander's medium, the virulence was decreased and could be used as an immunizing agent for chickens by atomizing the vaccine. This method of virus production maintained antigenicity as indicated by the hemagglutination inhibition (HI) tests on sera of chickens receiving this vaccine and a solid immunity in the vaccinated chickens to a challenge dose of 2×10^5 minimum infective doses of virulent virus. Although no respiratory symptoms were observed, some mortality (6.6%) and nervous symptoms were seen in chickens.

The successful intracerebral transmission of NDV through Syrian hamsters was reported by Reagan _et al_. (1947). This hamster brain virus could be neutralized by immune chicken serum as shown by tests in embryonating eggs. Later the same authors (1948) reported that continued intracerebral hamster passage of the virus had resulted in decreasing pathogenicity for chickens. The twenty-ninth intracerebral hamster passage virus produced typical NCD in approximately 10 per cent of the vaccinated birds. Less than 2 per cent of the birds showed symptoms when inoculated with the forty-ninth virus passage.

Beaudette (1948) described the disadvantages in using the attenuated live virus vaccine. Some systemic reaction and some mortality were observed following their use. The possibility of transferring other infectious agents from the incubated eggs, which were used in vaccine production, should also be taken into consideration. Moreover the use of attenuated live virus vaccine produced a mild infection in the flock which might be considered as a source of infection for non-vaccinated birds.

Zagar and Pomeroy (1950) found that following vaccination with commercial NCD live vaccine, NDV could be isolated from yolk material up to 10 days after vaccination. In the same vaccinated group they found a drop in production, and off-color, malformed and soft-shelled eggs.

C. Serological Tests

The evaluation of the efficiency of the NCD vaccines was done by immunization test. The birds were vaccinated with varying amounts of NCD vaccine and after 16 days were challenged with virulent NDV. At the time of the immunization test, serological tests were also performed to measure the antibody content of the sera.

There are two methods of measuring the content of antibodies against NCD in sera: the hemagglutination inhibition test and the serum neutralization test. Comparison was made of the antibody content in sera of birds taken before

vaccination, in sera of birds taken after vaccination and also of the antibody content in the sera of the non-vaccinated control birds. The efficiency of the vaccines was then evaluated by: (1) The protective effect of the vaccine against the challenging dose of virulent NDV, and (2) The increase in the antibody content of the vaccinated group.

1. Hemagglutination (HA) and Hemagglutination Inhibition (HI) Tests

Hirst (1941) found that when allantoic fluid infected with influenza virus was mixed with washed, normal chicken red blood cell suspensions, agglutination of the cells occurred within 5 to 20 minutes. The red blood cells sedimented rapidly and formed a characteristic ragged, granular pattern on the bottom of the tube. In contrast, when cells were mixed with uninfected allantoic fluid no hemagglutination occurred and the cells settled out slowly forming a sharp, round disc at the bottom of the tube. He also reported that hemagglutination was inhibited with influenza antiserum, and that the inhibition was specific for the homologous strain of virus.

The same phenomenon was discovered also by McClelland and Hare (1941), who showed that human and guinea pig red blood cells were also agglutinated by influenza virus.

Burnet (1942) reported the ability of NDV to agglutinate chicken red blood cells in a manner similar to that shown by influenza virus. A modification of the Hirst test, as suggested by Lush (1943), was used in reading the results. He also

reported the agglutinability of NDV on red blood cells of man, the guinea pig, mouse, sparrow and frog. Hemagglutination inhibition by specific NCD immune serum was also demonstrated.

Lush (1943) found that the reading, as described by Hirst (1942), was not satisfactory when used with NDV, because the virus affected only a small proportion of the red blood cells and did not reduce the density of the suspension sufficiently to permit the use of Hirst's technique. Therefore, the pattern of the agglutinated cells was used instead. One twenty-five hundredth of a milliliter of virus suspension with an equal volume of 2 per cent red blood cells suspension was used in the test. The results were read after incubation at room temperature for 2 hours and were recorded as 4 +, 3 +, 2 +, 1 +, ±, and 0, representing maximum to no agglutination. She also reported that by using the hemagglutination inhibition test, it was shown that no serological relationship between fowl plague and NDV is existed.

Salk (1944) described a simplified procedure for titrating the hemagglutinating capacity of influenza virus and the corresponding antibody. He used a 0.25 per cent red blood cell suspension made from a 10 per cent stock preparation stored at 4°C. Five-tenths milliliter of virus dilution was added to 0.5 ml. of 0.25 per cent red blood cell suspension. The readings were made after 1½ to 2 hours incubation at room temperature. In complete agglutination (+) the clumps of cells settled to the bottom of the tube with a dispersed

pattern rather than a compact pattern. A negative pattern (−) consisted of a central, sharply demarcated disc. Irregular clumps of cells associated with a halo of finely aggregated or unagglutinated cells represented an intermediate reaction (±). The end-point was the highest dilution of the virus producing maximal hemagglutination and the titer was expressed as the reciprocal of the final dilution of virus after the addition of red cells suspension. In the HI test the serum dilutions were prepared directly in the red blood cell suspension. Two hemagglutinin units of virus in 0.5 ml. of virus suspension were mixed with 0.5 ml. of red cell-serum dilution. The readings were made as in the HA tests. The HI titer of the serum was the highest dilution of serum producing complete inhibition of hemagglutination. Salk also noted that the titers of the agglutinin of several samples of infected allantoic fluid were essentially the same when tested with different batches of red cells, provided that the cells were carefully washed and suspended in the same way.

Burnet _et al_. (1945) showed that there was a difference between destruction of infectivity and hemagglutination activity of NDV. After heating at 60°C for 15 minutes the infectivity titer of the virus was about 1 to 10^8 of the original titer, whereas the hemagglutination titer was still 50 per cent of the original titer. At 65°C for 15 minutes the hemagglutination activity was destroyed completely. The addition of formalin in concentration of 0.1 to 0.25 per cent of the

virus destroyed its infectivity without changing the HA titer. It was shown by filtration that smaller particles or those less readily adsorbed by the filter than the virus, were responsible for a small but definite portion of agglutinating activity. Sensitization of chicken red blood cells by either NDV or influenza virus made the cells unagglutinable by NDV. The viruses in the influenza group could be arranged in a linear series, so that red blood cells sensitized by a given virus were agglutinated by the succeeding virus but not by the preceding virus. The series, in abbreviated form, was mumps, Newcastle disease, most influenza A strains, influenza B and swine influenza. Further work by Burnet et al. (1946) demonstrated that this was also true for human type O red blood cells.

Burnet et al. (1946) and Burnet and Anderson (1946) reported that red blood cells treated with amniotic or allantoic fluid preparations of NDV developed a new antigenic property. This new property produced agglutination in high titer, either by experimental NCD immune serum or by most sera from recent cases of infectious mononucleosis in man. They explained that an agent other than the virus (cell sensitizing agent), produced during the growth of NDV in chick embryos, was responsible for the changed character of the cells by adsorption to their surface.

Brandly et al. (1946, b) applied the Hirst phenomenon, as modified by Lush (1943) to identify NDV in infected amnioallantoic

fluid and found indifferent results. Satisfactory results were obtained after the red cell suspension was reduced from 2 to 1 per cent or less and incubated at room temperature (25°C) rather than at ice box temperature (6°C). The readings were taken at 15, 30 and 45 minutes instead of at 2 hours. They further reported that the difference between NDV and fowl plague virus could be determined by the HA test on various specific erythrocytes. NDV agglutinated swine red cells only slightly but plague virus clumped them completely. Rabbit red cells were agglutinated by NDV from one-fourth to one-half the degree obtained with chicken red cells but plague virus agglutinated these cells to a titer comparable with that of other species tested. It was found also that the HA titer of the NDV was retained well after treatment with heat, ultra violet ray and formalin sufficient to destroy infectivity.

Cunha et al. (1947) reported that the intensity of the reaction was too low when the Lush's modification of the test was used. Satisfactory results could be obtained when the tests were carried at 23° to 27°C. The dilutions of virus were made in buffered saline (pH 7.4) and a final red blood cell concentration of 1 per cent was used. The end point dilution was the tube that matched the standard tube of 0.63 per cent cell concentration. They also reported that the hemagglutinative capacity of NDV was closely associated either with the virus itself, as indicated by infectivity measurements, or with material consisting of particles of sedimentative

character closely resembling those of the virus. A factor in normal allantoic fluid was found to have an inhibitory action on the HA test. Variation in the ratio of the hemagglutinative activity and infectivity was greater than in influenza virus. This variation was due to the relatively low capacity of NDV to agglutinate red blood cells and greater difficulty in controlling the conditions of the reaction.

Florman (1947) showed that HA test with NDV was markedly affected by temperature. At room temperature the reactions were difficult to read, especially with low dilutions of virus. This effect resembled a prozone reaction and disappeared when the tests were carried out at 4°C. Florman thought that the low HA titer was due to rapid elution of the virus at room temperature so that agglutination was masked in lower dilutions of virus. At 4°C NDV appeared to be adsorbed more completely by and to elute less rapidly from chicken red cells than at room temperature. Although the end-point of hemagglutination at 4°C was lower than at 37°C. Anderson (1947) explained that higher titer of virus at 37°C was considered to be due to the disruption of virus aggregates in contact with red blood cells with ultimate liberation of single virus particles.

Anderson (1947) reported the hemagglutinability of the fresh red blood cells by the NDV stabilized red blood cells as described by Burnet and Anderson (1946). This agglutination was caused by an agglutinin on stable cells. He also stated that all manifestations of NDV action on red blood

cells were essentially due to the virus particles *per se*. NDV in infected embryonic fluid was found to be nonhomogenous and larger units of NDV can be segregated. After hemagglutination, elution occurred by the partial disruption of the aggregates and one virus particle remained attached to the red blood cell. This remaining particle could leave the red cell only by transfer to another cell, not by elution into the supernatant fluid. Its presence on the red cells rendered the cells susceptible to agglutination by NCD immune serum and functioned as the retained hemagglutinin. The agglutinin on stable cells was neutralized by NCD immune serum. The serum titers corresponded closely with the direct HI titers. In NCD immune serum, the same antibody molecule seemed to function either as an anti-hemagglutinin or as agglutinin for sensitized cells. Infectious mononucleosis immune serum, which caused hemagglutination of NDV sensitized human cells, had no action as an anti-hemagglutinin.

The influence of the route of inoculation of NDV on development of virus hemagglutinin and of virus LD 50 titer for embryo had been reported by Hansen *et al*. (1947). Hemagglutinin developed most rapidly in embryonating eggs inoculated by intravenous route, yolk sac and allantoic route respectively. Materials from embryos inoculated upon the chorioallantoic membrane failed to show HA titer until 24 hours after inoculation. HA titer showed a progressive increase from the first appearance of the hemagglutinins until

the death of the embryo. Normal hemagglutinin was found in both normal and infected yolk sac membranes, and thus masked the virus hemagglutinative activity. This normal hemagglutinin was inhibited by the addition of either normal serum or NCD immune serum. They also reported that virus suspensions having an embryo LD 50 titer of 10^{-5} or less failed to produce demonstrable hemagglutination. Suspensions with an embryo titer of 10^{-6} had corresponding hemagglutinin titers ranging from undiluted or 1:5 to 1:160. With an embryo titer of 10^{-8} the agglutination range was from 1:160 to 1:1,280. The embryo LD 50 titration was reported more sensitive in detecting small quantities of virus than the HA test.

Brandly _et_ _al_. (1947) reported that the storage of allantoamniotic fluid infected with NDV in the unfrozen state for an interval of several months had not been found to result in appreciable loss of HA titer. They confirmed the variations in sensitivity among the cells of different birds. The variation in hydrogen ion concentrations within the range of pH 6 to pH 8 did not cause an alteration in agglutination, velocity, pattern or titers. Comparisons were made between the "alpha" procedure wherein successive dilutions of virus are admixed with a fixed amount of serum and the "beta" procedure wherein serum is titrated by dilutions against a selected small quantity of virus. The "alpha" procedure had been found somewhat more economical than the "beta" procedure from the standpoint of equipment, materials and manipulations.

The latter, however, was more satisfactory for quantitative titrations. The agreement of 93 per cent between the results of the "alpha" and "beta" procedure, indicated that relatively high accuracy could be obtained by each procedure. A HI titer of 80 for "alpha" procedure was considered to be positive. All sera which by the "beta" procedure failed to inhibit 10 HA units when diluted 1 in 5 were considered to be negative. They further stated that during the ascending period, generally, the first 3 weeks following a simulated natural infection or following vaccination, a good agreement of HI and serum neutralizing titers might be obtained. During the descending phase, these titers might show either correlation or divergence. The HI antibodies were observed to disappear first. Revaccination of birds increased both serum neutralizing and HI antibody.

Osteen and Anderson (1948) reported that a diagnostic HI titer was obtained from the test birds at an earlier time than was obtained by the serum neutralizing method. This was true when using a diagnostic titer of 80 or more for the HI test and 1,000 or more neutralizing doses for the serum neutralizing test. Dilution of positive serum with negative serum in a ratio of 1:9 failed to lower the serum neutralizing titer, but with 1:19 lowered the tenfold titer to the next measurable level. He further mentioned that serious error because of dilution with serum from non infected bird was improbable due to the high morbidity in NCD. Therefore, a composite sample of 10 birds in a flock of 1,000 in number could be used for

serum neutralization test. The HI test was equally accurate and reliable as the serum neutralization test if the test was conducted carefully.

Beach (1948) described the application of HI test in the diagnosis of avian pneumoencephalitis (NCD). "Beta" procedure was used and the tests were read from the pattern of agglutination as described by Lush (1943) by placing the rack above the mirror. He found that HI titers were extremely variable but ranged higher with sera of birds which had been hyperimmunized or had survived severe field infection. The titer, however, was not always correlated with the severity of exposure to the virus or with the degree of the disease. Sera from chickens which had been vaccinated against pneumoencephalitis were negative to the test, suggesting that active virus might be required for the production of HI antibodies. The specificity of the HI tests had been confirmed. A close relation was found between the ability of serum to inhibit the agglutination of red blood cells and to neutralize the virus as tested by chicken inoculation. Sera having the same virus neutralizing titer, however, had a wide range of HI titer.

2. Serum Neutralization Tests

Doyle (1927) first demonstrated that serum from NCD immune chickens would neutralize or inactivate the NDV as shown by chicken inoculation.

Burnet and Ferry (1934) reported that NDV multiplied rapidly in embryonated chicken eggs. Using the chorioallantoic

membrane method the virus caused death invariably within 48 hours. The developing embryos could, therefore, be used as an indicator of the presence or absence of a lethal dose of virus. Later Burnet (1942) reported that NDV could be propagated in eggs by various methods. Inoculation of the virus into the allantoic cavity of the embryonating eggs, the virus multiplied freely and caused specific death of the embryos. Although the characteristics of NDV resembled those of the influenza virus, i.e. hemagglutination and multiplication in eggs, there was no serological relation between these two viruses.

Keogh (1937) found that NDV could be neutralized by its corresponding immune serum. This was shown by mixing undiluted serum with the virus in vitro and inoculating it upon the chorioallantoic membrane of embryonating eggs. The serum protected the embryos against 1,000 embryo lethal doses of virus. When the inoculation of virus was done prior to injection of the serum, the eggs invariably died, even when the serum was much more than sufficient to neutralize the virus, though the eggs survived longer than the controls with minimal infective dose of virus without serum. Keogh also observed that there was no significant difference by either egg or chicken inoculation.

Beach (1942) demonstrated the neutralizing effect of NCD immune serum. An equal part of immune serum was mixed with 100 minimum infective doses of virus. The mixture was inoculated

into chickens after 4 hours at refrigerator temperature. The chickens inoculated with serum-virus mixture were not infected, while the control birds succumbed. The less severe infection produced in chickens by inoculating with a mixture of serum diluted 1 in 10 and virus suspension indicated partial neutralization of virus had occurred. Undiluted normal serum or more diluted immune serum could not neutralize the infectivity of the virus.

Minard and Jungherr (1944) developed a method for the quantitative neutralization test of avian pneumoencephalitis virus in 8 to 10 days embryonating eggs. The method consisted of mixing equal part of dilutions of virus suspensions in broth and undiluted serum. The mixture was incubated at 37°C for 1 hour and 0.1 ml. of the mixture was inoculated into the allantoic cavity of embryonating eggs. The eggs were reincubated for 4 days. The majority of the infected embryos were found dead within 48 to 72 hours after inoculation. Embryos surviving this period were usually found to live and hatch normally. The neutralizing doses were expressed as the reciprocal of the difference between the titers of the control and the serum-virus mixtures. The dilution at which one-half of the inoculated embryos survived was taken as the critical titer. They also stated that parallel titrations of 2 sera by intramuscular injection in chicks and allantoic cavity injection in embryos gave similar results.

In 1945 the Committee on Transmissible Diseases of Poultry of the U. S. Livestock Sanitary Association considered the serum neutralization test to be diagnostic if the tested serum neutralized at least 1,000 embryo lethal doses of NDV.

Brandly _et al_. (1947, b) reported that neutralization tests in embryonating eggs were satisfactory and reproducible within tenfold titer limits. The standard procedure was adding undiluted serum to an equal quantity of various dilutions of virus to the estimated end point infective titer. Control titrations of the virus were always made in parallel. The mixtures were incubated for 30 to 90 minutes at room temperature prior to injection. There was no significant difference in titer within 24 hours and between the temperature range from 23° to 38°C. The results were evaluated by 50 per cent mortality during a subsequent 4 days' incubation period of the eggs. It was found that virus titers in chickens and in eggs via several routes are virtually identical. The highest neutralization values were obtained in chickens, somewhat lower titers were obtained with the allantoic chamber. The neutralization titers of the serum did not change when the serum was treated with 0.02 to 0.01 per cent of Merthiolate or 1 per cent phenol.

III. MATERIALS AND METHODS

A. Newcastle Disease Virus

NDV strain 54-55-18 was isolated in the Department of Microbiology and Public Health, Michigan State University on June 22, 1954 from a naturally infected chicken. The brain from an infected bird was ground with sand and suspended in 2 ml. broth, with 10,000 units of penicillin and 10,000 mu. of streptomycin per ml. This suspension was inoculated into the allantoic sac of 3 ten-day embryonating eggs and reincubated at 37°C. The virus killed 2 embryos within 72 hours after inoculation and the other one at 96 hours. The allantoic fluid was harvested from the embryos dead at 72 hours.

B. Preparation of Virus Suspension

White Leghorn eggs obtained from a flock free of NCD were used in all studies. The embryos were from 8 to 10 days old, but in each test all embryos employed were of the same age. The eggs were incubated in an electric, forced draft incubator at 37°C. Infected allantoic fluid was passed through 2 sets of 20 embryonating eggs serially by the allantoic route and reincubated at 37°C. The virus killed all the embryos within 48 hours after inoculation. The pooled allantoic fluid was kept frozen at -25°C for use for further passages.

One hundred 10-day embryonating eggs were used for the propagation of the stock virus. The eggs were transilluminated for selection of an area on the chorioallantoic membrane that was free from large blood vessels. A small hole was drilled through the shell without piercing the shell membrane with a small drill attached to the chuck of an electric motor. Another hole was drilled through the shell over the top of the air cell. Before inoculation, tincture of metaphen was applied over the holes and allowed to dry. The shell membrane over the air cell was punctured with a sterile dissecting needle to allow equalization of pressure within the egg when the inoculum was injected into the allantoic cavity and to prevent leakage of the inoculum from the site of injection.

The eggs were inoculated via the allantoic cavity with a tuberculin syringe fitted with a 27-gauge, one-half inch needle through the hole on the side of the egg. One-tenth ml. undiluted infected allantoic fluid, from the third egg passage, was used for each egg. The holes in the shells were sealed with melted paraffin and the eggs returned to the incubator. All eggs were candled every 24 hours to determine death of embryos. Death of embryos in the first 24 hours were considered to be due to injury. The embryos dead after 24 hours were removed from the incubator and chilled in the refrigerator at 4°C for 4 to 16 hours to diminish possible hemorrhage from ruptured vessels when the allantoic fluid was collected.

To harvest the fluid the shell over the air cell was swabbed with tincture of metaphen, allowed to dry and the shell was cracked and removed with sterile forceps. Sterile scissors and forceps were used to cut the shell membrane and allantoic membrane underneath the air cell. Allantoic fluid was then removed. The allantoic fluid from 4 to 5 eggs was pooled in the same test tube and tested for bacterial sterility in thioglycollate medium. The allantoic fluids were then frozen. Tubes showing bacterial contamination were discarded. The contents of the bacteria-free tubes were pooled in a sterile Erlenmeyer flask and then distributed in 10 ml. amounts into glass vials and sealed. The vials were then stored at -25°C until needed. This NDV suspension was labeled 54-55-18 P 4.

Titration for virus infectivity was done by inoculation of serial tenfold dilutions of virus suspension into the allantoic sac of 8-to 10-day embryonating eggs as previously described, 5 eggs per dilution were used with 0.1 ml. inoculum per egg. The eggs were incubated for 7 days after inoculation. The mortality rates were recorded and the LD 50 was determined by the method of Reed and Muench (1938). The titer of NDV suspension 54-55-18 P 4 was found to be $10^{-9.5}$ LD 50, which means that the virus suspension contained $10^{9.5}$ embryo lethal doses (LD 50) in 0.1 ml. The HA titer of the virus was 1:320.

C. Preliminary Experiment

B.P.L. used throughout these experiments was supplied by Dr. Thomas L. Grestiam of the B. F. Goodrich Company, Breckville, Ohio. To facilitate the measurement of B.P.L., especially when small amounts of infected allantoic fluid were used, B.P.L. was diluted 1 in 10 with sterile distilled water before use in every experiment. The percentage of B.P.L in the experiment was the percentage of concentrated B.P.L. in the final virus mixture.

Varying amounts of B.P.L. (0.025, 0.05, 0.1, 0.2 and 0.3%) by volume were sprayed on the virus suspension in a Petri dish. This was done by drawing a drop of the B.P.L. solution into a one-fourth ml. syringe with a 27-gauge needle. The content of the syringe was expelled and then the plunger of the syringe was rapidly forced back and forth to produce a fine spray of B.P.L. The dish was rotated continually. The mixture was transferred to a test tube and incubated at 37°C for 2 hours together with a virus suspension not containing B.P.L. as control. After the end of the two-hour period, the mixtures were then inoculated into the allantoic sac of 10-day embryonating eggs, 0.1 ml. per inoculum and 5 eggs per B.P.L. dilution. The eggs were incubated for 7 days after inoculation before being discarded. The infectivity test of the control virus was done to determine the titer of the virus after incubation at 37°C for 2 hours.

The B.P.L.-virus mixtures were then tested for hemagglutinability to determine if B.P.L. changed that characteristic.

TABLE I

VIRICIDAL EFFECT OF BETA-PROPIOLACTONE ON NEWCASTLE DISEASE VIRUS INFECTIVITY TEST IN EGGS

% B.P.L.	Mortality Rate #
0.025	0/5
0.05	0/5
0.1	0/4
0.2	0/4
0.3	0/5
0.0 Control	5/5

Numerator No. dead / Denominator No. inject

TABLE II

EGG INFECTIVITY OF NEWCASTLE DISEASE VIRUS BEFORE AND AFTER INCUBATION FOR TWO HOURS AT 37°C

	Virus Dilutions						Virus Titer
	Und.	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	LD 50
Before Incubation	5/5	5/5	5/5	5/5	3/5	2/5	9.5
After Incubation	5/5	5/5	5/5	5/5	2/4	2/5	9.43

Numerator No. dead / Denominator No. injected

TABLE III

HEMAGGLUTINATION TEST OF NEWCASTLE DISEASE VIRUS TREATED WITH BETA-PROPIOLACTONE

Virus + % B.P.L.	Und.	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	HA Titer
Control No B.P.L.	+	+	+	+	+	+	+	+	-	1:320
0.025	+	+	+	+	+	+	+	+	-	1:320
0.05	+	+	+	+	+	+	+	+	-	1:320
0.1	+	+	+	+	+	+	+	+	-	1:320
0.2	+	+	+	+	+	+	+	+	-	1:320
0.3	+	+	+	+	+	+	+	-	-	1:160

+ Hemagglutination

- No hemagglutination

D. Preparation of Newcastle Disease Vaccine

The frozen NCD infected allantoic fluids were thawed and centrifuged at 2,000 r.p.m. in horizontal centrifuge just prior to use to remove any gross particles. From several preliminary experiments it was found by inoculation of embryonating eggs that 0.025 per cent by volume of B.P.L. destroyed infectivity of NDV without changing the hemagglutination titer. Two hundred ml. of allantoic fluid were transferred into a sterile 500 ml. Erlenmeyer flask with rubber stopper. Five-tenths ml. of an aqueous solution (1:10) of B.P.L. was added by sterile serological pipette to the virus suspension drop by drop with constant shaking. The final concentration of B.P.L. in the virus suspension was 0.025 per cent. After addition of B.P.L. the mixture was incubated at 37°C for 2 hours. The vaccine thus prepared, was used for inoculation of the experimental chickens immediately after incubation.

E. Vaccination

White Leghorn chickens about 10 months old were used in these experiments. All birds were obtained from an apparently disease-free flock and were kept for observation for the indication of any disease for about 10 days prior to the experiment. The birds were all apparently healthy at the beginning of the experiment. Of the 82 birds obtained, 43 birds were

used for vaccination and 39 birds were used as non-vaccinated controls. The two groups of birds were separated in 2 isolation units. Prior to the experiment the birds were marked individually by wing band and were bled from the heart. The sera were separated and kept frozen as prevaccination sera for the vaccinated group and normal sera for the control group.

The birds receiving vaccine were divided into 4 groups. Each group was inoculated with the same vaccine but in different dosage. Group 1 consisted of 13 birds and received 0.5 ml. of vaccine. Groups 2, 3 and 4 consisted of 10 birds each and received 1.0, 1.5 and 2 ml. of vaccine respectively. All injections were intramuscular into pectoral muscle, using 2 ml. syringe with a 1-inch 22-gauge needle. The control birds received no injections.

None of the vaccinated birds showed symptoms of NCD or other untoward effects of injection of the B.P.L. inactivated NCD vaccine. All the vaccinated birds were bled from the heart again on the sixteenth day after vaccination just before challenging with NDV. The sera were separated and kept frozen as postvaccination sera.

Both vaccinated and non-vaccinated control birds were challenged with the same lot of virus as that used to make the vaccine (54-55-18 P 4). The amount of the virus introduced into each bird was equal to 2 x $10^{9.5}$ embryo lethal doses. Two-tenths milliliter of full strength virus was inoculated into the pectoral muscle of each bird.

F. The Bactericidal Activity of B.P.L. on *Salmonella pullorum*

The incidence of *Salmonella pullorum* in eggs from infected hens had been demonstrated by Mallman and Moore (1936). Therefore, the transmission of this organism would be possible if eggs from pullorum-infected hens were used in vaccine production. The purpose of this experiment was to determine if *Salmonella pullorum* would be killed by B.P.L. in the concentration that did not alter the antigenicity of NDV as shown in the vaccination experiment.

Salmonella pullorum used in this experiment was the strain 54-55-552, isolated from a naturally infected chicken in the Department of Microbiology and Public Health, Michigan State University. This experiment was done in three parts.

1. The Action of 0.025 Per Cent B.P.L. on *Salmonella pullorum* Mixed with Newcastle Disease Virus Suspension

Salmonella pullorum was grown on a nutrient agar slant for 48 hours at 37°C. The growth was removed from the agar slant with 3 ml. of sterile saline solution and was transferred into a small test tube. The bacterial suspension was shaken vigorously for several minutes to distribute bacteria evenly in the suspension. The standard plate count method was used in determining the number of *Salmonella pullorum* in the bacterial suspension and it was found to contain 2,000,000,000 organisms in 0.1 ml. One-tenth milliliter of *Salmonella pullorum* suspension was added to a 10 ml. suspension of NDV lot 54-55-18 P 4. An

aqueous solution of B.P.L. (1:10) was added to make a final concentration of 0.025 per cent. The mixture was made in the same way as described under the preparation of NCD vaccine. The mixture then was shaken for a few minutes and incubated at 37°C for 2 hours. The vaccine was used immediately after incubation.

The vaccine was tested for sterility. One loopful of the vaccine was transferred into a tube of nutrient broth and incubated for 48 hours at 37°C. After 48 hours' incubation, the growth in the nutrient broth was observed and was transferred to Kligler's medium and carbohydrate media. After 48 hours' incubation of the differential tests, *Salmonella pullorum* was confirmed.

Ten White Leghorn chickens were used in this experiment and were obtained from the same source and were kept under the same conditions as described in the preceding experiment. The birds were bled from the heart before vaccination and the sera separated and frozen for serological test to be done later. Five-tenths milliliter of NCD vaccine containing *Salmonella pullorum* was inoculated into the pectoral muscle of each bird. No symptoms of either NCD or pullorum infection were observed during the observation period. Seventeen days after vaccination, all birds were bled from the heart. The sera were separated and kept frozen as postvaccination sera. The birds were killed; post mortem examination revealed no gross change indicating either NCD or pullorum infection.

2. The Action of Different Concentrations of B.P.L. on *Salmonella pullorum*

Salmonella pullorum was seeded into 200 ml. nutrient broth in a 500 ml. Erlenmeyer flask and incubated at 37°C for 18 hours. Standard plate count showed that the number of organisms was 150,000,000 organisms per ml. At the end of 18 hours, 10 ml. of the culture was distributed into each of 8 tubes. An aqueous solution of B.P.L. (1:10) was added into each tube so that the final concentration of B.P.L. varied from 0.025, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 per cent respectively. One tube was used as a control and contained no B.P.L. The tubes were then incubated at 37°C for 2 hours. To dilute the B.P.L. 0.1 ml. of each tube was transferred into a tube of nutrient broth and incubated for 15 days at 37°C. The bactericidal effect of B.P.L. was shown by the absence of growth in the subcultured tube after 15 days' incubation.

TABLE IV

BACTERICIDAL EFFECT OF B.P.L. ON *SALMONELLA PULLORUM*

%B.P.L.	Growth
0.025	+
0.05	+
0.1	-
0.2	-
0.3	-
0.4	-
0.5	-
Control no B.P.L.	+

\- No growth in nutrient broth after 15 days' incubation.
\+ Growth after 15 days' incubation.

3. The Effect of 0.1 Per Cent B.P.L. on the Antigenicity of Newcastle Disease Virus

The information given from the experiment on the action of different concentration of B.P.L. showed that *Salmonella pullorum* was killed when mixed with B.P.L. at the final concentration of 0.1 per cent. This experiment was to determine whether or not B.P.L. at 0.1 per cent concentration would destroy the antigenicity of NDV.

Ten ml. of NDV suspension (lot 54-55-18 P 4) were mixed with 0.1 ml. of aqueous B.P.L. (1:10) as previously described. The concentration of B.P.L. was 0.1 per cent. The B.P.L.-virus mixture was incubated for 2 hours at 37°C and was immediately thereafter inoculated into 5 chickens.

Five White Leghorn chickens about 1 year of age were used in this experiment and each was kept separately in a wire cage. The prevaccination sera were obtained as in the previous experiment. One-half milliliter of the vaccine was injected into the pectoral muscle of each bird. All birds remained apparently healthy after the vaccination. The post-vaccination sera were obtained on the fifteenth day following vaccination and were stored in a frozen state. The birds were then challenged with 0.2 ml. of undiluted Newcastle disease virus suspension (lot 54-55-18 P 4) intramuscularly. The challenging virus dose was from the same lot and amount as used to challenge the original vaccine test.

G. The Effect of B.P.L. On Infectious Bronchitis Virus

Infectious bronchitis virus (strain V 114 D) was used in this experiment. One-tenth of a milliliter of infected allantoic fluid was inoculated into the allantoic cavity of each of twenty 10-day embryonating eggs and reincubated at 37°C. All embryos were dead within 24 hours after inoculation. The eggs were chilled and the allantoic fluids were harvested as described in the preparation of NDV suspensions. The allantoic fluids were pooled together and kept frozen for further use. Five milliliters of virus suspension were distributed into each of 6 test tubes. An aqueous solution of B.P.L. (1:10) was added to 5 of the 6 tubes so that the final concentration of B.P.L. in each tube was 0.025, 0.05, 0.1, 0.2 and 0.3 per cent respectively. The sixth tube received no B.P.L. and was the virus control. The mixtures then were incubated for 2 hours at 37°C together with the control virus. After incubation the mixtures were immediately inoculated into allantoic cavity of 10-day embryonating eggs, using 0.1 ml. per egg and 5 eggs per dilution. The inoculated eggs were reincubated for 7 days. The mortality of the embryos dead within 12 hours after inoculation were regarded as non specific.

The infectivity titer of infectious bronchitis virus was determined by the same method as described for NDV and was found to be $10^{6.12}$ LD 50 per 0.1 ml. of inoculum.

TABLE V

VIRUCIDAL EFFECT OF B.P.L. ON INFECTIOUS BRONCHITIS VIRUS

% B.P.L.	Mortality Rate #
0.025	0/5
0.05	0/5
0.1	0/5
0.2	0/4
0.3	0/5
Control No B.P.L.	5/5

\# Embryo dead / Embryos inoculated

H. The Effect of B.P.L. on Infectious Laryngotracheitis Virus

Infectious laryngotracheitis virus (strain 11-3-54) in chorioallantoic membrane was used as the source of the virus. The membrane was a pooled harvest from 12-day egg passages. The infected membranes were ground with sterile sand in a mortar with pestle and suspended with a small amount of nutrient broth. Penicillin (10,000 units) and streptomycin (10,000 mu. per ml. of nutrient broth) were added to the suspension to prevent and inhibit bacterial growth. The final suspension was deposited on the chorioallantoic membrane in the artificial air cell of 12-day embryonating eggs as described by Beveridge and Burnet (1946). Two-tenths of a milliliter were used to inoculate each egg. The eggs were reincubated at 37°C and candled at 24-hour intervals after inoculation. Embryo mortality during 24 hours after inoculation was considered as non specific. On the fourth day after inoculation, the eggs

were candled and infected chorioallantoic membranes were removed. The harvested membrances were stored in a frozen state at -25°C.

The infectious laryngotracheitis virus suspension was prepared as described above. Five milliliters of virus suspension were distributed into each of 6 test tubes. An aqueous solution of B.P.L. (1:10) was then added in different quantities to the first 5 tubes to make the final concentration of B.P.L. 0.025, 0.05, 0.1, 0.2 and 0.3 per cent respectively. The mixtures, together with the sixth control tube of virus suspension without B.P.L., were incubated at 37°C for 2 hours. After incubation, two-tenths milliliter of the mixture from each tube was deposited on the chorioallantoic membrane in the artificial air cell of 30 twelve-day embryonating eggs, using 5 eggs per tube. On the fourth day after inoculation, the chorioallantoic membranes were harvested and examined for the grayish, thickening, yellow plaque as the criterion of growth of the infectious laryngotracheitis virus. Infectious laryngotracheitis virus generally does not kill the embryo.

TABLE VI

VIRUCIDAL EFFECT OF B.P.L. ON INFECTIOUS LARYNGOTRACHEITIS VIRUS

% B.P.L.	Lesions
0.025	-
0.05	-
0.1	-
0.2	-
0.3	-
Control No B.P.L.	+

- No thickening of the chorioallantoic membrane.

+ Thickening of the chorioallantoic membrane, characteristic lesions of Infectious Laryngotracheitis virus.

I. Serological Tests

Blood samples were drawn with aseptic precaution. Pre-vaccination bleedings were done just prior to the vaccination. Post-vaccination bleedings were done prior to the challenging by untreated virulent virus. About 15 ml. of blood were drawn from each bird by cardiac puncture, using 1¼-inch 18-gauge needle attached to a 10-ml. glass syringe. The blood was allowed to clot for about one-half hour and then the tubes were wrung with sterile wood applicators. The tubes were stored in a refrigerator at 4°C for 4 to 5 hours. Finally the tubes were centrifuged in a horizontal centrifuge at 2,000 r.p.m. for 10 minutes to separate the sera. The sera were removed from the clot and stored in a freezer at -25°C.

Some of the Newcastle disease vaccinated birds were bled and sacrificed at the end of the second and third months after

the challenging virus dose. The birds were then examined for evidence of Newcastle disease. The blood sera collected from these birds were frozen and held for serological examination.

The paired sera samples were tested for the antibody content by:

1. Hemagglutination-inhibition tests.
2. Serum-neutralization tests in embryonating eggs.

1. Hemagglutination (HA) and Hemagglutination Inhibition (HI) Tests

(1) Virus: Blood-free Newcastle disease virus suspension (lot 54-55-18 P 4), which had been previously prepared and kept frozen at -25°C were used throughout these tests.

(2) Red blood cells: Blood was drawn by cardiac puncture from chickens. The tubes receiving the blood contained 1 ml. of 2 per cent sodium citrate in saline solution for each 9 ml. of blood. The blood was mixed with the anticoagulant thoroughly to prevent clotting. The cells were then sedimented by centrifugation at 1600 r.p.m. for 8 minutes and the supernatant plasma removed. The cells were then washed 3 times with 10 volumes of saline solution. The final centrifugation to pack the cells was for 10 minutes at 1,000 r.p.m. After washing, the packed cells were stored in the refrigerator at 4°C. The cells were found satisfactory for use in the test for as long as 6 days (Hirst, 1942; U.S.D.A., 1946). A 0.5 per cent suspension of red blood cells in saline solution was freshly prepared immediately prior to use in the tests.

(3) Saline solution: Sterile chemically pure 0.85 per cent sodium chloride solution was used throughout the tests.

(4) Serum: The serum to be tested was diluted 1 in 5 with saline solution or was diluted as indicated in the result.

The tubes used in the test were chemically clean, round bottom glass tubes (75 x 10 mm.). The virus suspension was thawed and centrifuged in a horizontal centrifuge to remove the insoluble substances at 2500 r.p.m. for 20 minutes. The supernatant fluid was removed and used for making virus dilution. From the preliminary experiment it was found that Newcastle disease virus suspension 54-55-18 P 4 produced maximum hemagglutination in a dilution of 1:320. Progressive two-fold dilutions of the virus from 1:5 through 1:640 were made in 0.85 per cent saline solution. Twenty-five-hundredths milliliter of diluted (1:5) serum was distributed into each tube of the hemagglutination inhibition test. In the hemagglutination test 0.25 ml. of saline solution was used instead of diluted serum. Twenty-five hundredths milliliter of virus dilution was transferred into parallel rows of tubes containing diluted serum or saline solution according to the test. The control tubes contained either saline solution and cells or serum and cells for the respective tests. The proctocols of the procedure used in hemagglutination inhibition and hemagglutination test are given in Table VII, page 46. The tubes were shaken and 0.25 ml. of a 0.5 per cent red blood cell suspension was added to each tube. The tubes were shaken again and were

incubated at room temperature of from 22°C to 27°C. Readings, the result of the tests, were taken at 15, 30 and 45 minutes. The earlier readings were made to detect hemagglutination which might occur in the low-dilution tubes and disappear before the final readings (45 minutes) were made. The reading was facilitated by viewing the tubes from the bottom of the rack through a mirror placed under the rack.

Maximum hemagglutination was exhibited as a uniform layer of agglutinated cells covering the bottom of the tube and was recorded as positive (+). In the control tube and in the tubes where there was no agglutination, the cells sedimented into a central, sharply demarcated red disc in the bottom of the tube and was recorded as negative (-). Irregular clumps of cells associated with a ring of finely aggregated cells were recorded as partial or slight agglutination (±).

Virus hemagglutination (HA) titer was the highest dilution of the virus which produced complete hemagglutination. This dilution was considered to contain 1 hemagglutinin unit. The number of hemagglutinating units in 0.25 ml. of the undiluted virus then was the reciprocal of the dilution of the virus.

The serum titer was the lowest dilution of the virus in which hemagglutination was completely inhibited. This dilution was considered to contain hemagglutination inhibition units equal to the number of hemagglutinin units in that dilution. The hemagglutination inhibition units in 0.25 ml. of undiluted serum were then computed and were expressed as the hemagglutination

inhibition titer of that serum. For convenience in calculation of the hemagglutination inhibition titer of the serum, the following formula could be used:

$$\frac{\text{Reciprocal of virus titer x dilution of serum}}{\text{Reciprocal of serum titer}} = \text{HI titer of serum}$$

A HI titer of 80 or more was considered to be diagnostic of previous vaccination or infection with Newcastle disease.

TABLE VII

HEMAGGLUTINATION AND HEMAGGLUTINATION-INHIBITION TESTS PROCEDURE

Material	Und.	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	Control
HA Test										
Virus dilution (ml.)	- - - - - 0.25 ml. per tube - - - - -									0
0.85% Saline solution (ml.)	- - - - - 0.25 ml. per tube - - - - -									0.5
0.5% Suspension r.b.c. (ml.)	- - - - - 0.25 ml. per tube - - - - -									0.25
HI Test										
Virus dilution (ml.)	- - - - - 0.25 ml. per tube - - - - -									0
Serum dilution (ml.)	- - - - - 0.25 ml. per tube - - - - -									0.5
0.5% Suspension r.b.c. (ml.)	- - - - - 0.25 ml. per tube - - - - -									0.25

2. Serum Neutralization Tests

Newcastle disease virus suspension (54-55-18 P 4) was used in performing serum neutralization throughout this study. Prior to use the virus suspension was thawed and centrifuged at 2500 r.p.m. for 20 minutes in a horizontal centrifuge to remove the insoluble precipitate formed by freezing. One thousand units of penicillin per ml. of nutrient broth were used in making virus dilutions to prevent possible contamination from either virus suspension or serum. Greater concentrations of penicillin have been used by Brandly *et al*. (1946, b) in serum neutralization tests without alteration of titers. From preliminary experiment it was found that the LD 50 titer of virus suspension was $10^{-9.5}$. Serial ten-fold dilutions of allantoic fluid containing the virus were prepared in nutrient broth from 10^{-1} to 10^{-10}. Separate pipettes were used in preparing each dilution. Equal parts of undiluted serum and diluted virus were mixed in separate tubes for each dilution of virus. To compensate for the increased dilution of the virus in the serum-virus mixture, the control virus dilution was mixed with an equal volume of broth. The serum-virus mixtures were incubated at room temperature for 30 minutes before being inoculated into eggs. The virus dilutions were freshly prepared prior to the test and the infectivity test in eggs was done in parallel.

One-tenth milliliter of the serum-virus mixture was inoculated into the allantoic sac of 8-to-10-day embryonating eggs by using a tuberculin syringe attached to a 27-gauge, ½-inch

needle. Five eggs were inoculated from each serum and virus dilution. The preparation of eggs was the same as described under the preparation of the virus suspension. A single syringe was used for each serum sample. Bacterial sterility tests were done on each preparation. After the eggs were inoculated, the holes in the shell were sealed with melted paraffin and reincubated for 7 days at 37°C. The eggs were candled daily. The criterion of viral infectivity was the mortality of the embryos. Mortality within the first 24 hours after inoculation was considered as non specific and was not included in the calculation of mortality rates.

The virus titer was the highest dilution of virus in which 50 per cent mortality of the embryos occurred and was considered as 1 embryo lethal dose. The number of embryo lethal doses in 0.05 ml. of the undiluted virus suspension was the antilogarithm of the reciprocal of the dilution.

The serum titer was considered to be the lowest dilution of virus in which the serum neutralized the virus to the extent that 50 per cent of the embryos survived. The difference between the logarithm of virus titer and logarithm of serum titer is the neutralization index, the antilog of which is the number of neutralizing doses in 0.05 ml. of undiluted serum.

For convenience in demonstrating the results both virus and serum titers were expressed as the negative logarithms of their respective values throughout this study.

IV. RESULTS

Preliminary tests indicated that beta-propiolactone (B.P.L.) is capable of destroying the infectivity of Newcastle disease virus. When concentrations of 0.025 through 0.3 per cent B.P.L. were added to the virus (Table I, page 31) and this mixture was then injected into embryonating eggs, no death due to infectivity occurred. It was also shown that the B.P.L. in these concentrations, was not toxic to the embryos. To make certain that the virus was not destroyed by the incubation period of 37°C for 2 hours, a control sample of virus alone was incubated at the same temperature for 2 hours. The results of this experiment are shown in Table II, page 31. The virus suspension contained LD 50 per cent titer of 9.50 before the inoculation period and dropped to 9.43 following the incubation treatment. No significant virus alteration was found to result from this treatment.

The effect of varying the concentration of B.P.L. on the hemagglutination property of Newcastle disease virus is illustrated in Table III, page 32. No alteration in the hemagglutination titer could be detected until 0.3 per cent concentration of the B.P.L. was used. The control material contained virus only and the virus with B.P.L. concentration through 0.2 per cent agglutinated erythrocytes at a 1:320 dilution. A 0.3 per cent concentration of B.P.L. in the virus suspension

reduced the hemagglutination titer to 1:160 which is within the accuracy of a tube test.

To determine what effect the B.P.L. would have upon the antigenicity of Newcastle disease virus, 5 chickens were vaccinated with Newcastle disease virus which had been treated with B.P.L. The concentration of B.P.L. was 0.1 per cent and the vaccine prepared in the same manner as has already been described. Sera were collected from the 5 birds prior to vaccination and 15 days after vaccination. The antigenic stimulation produced by the vaccine in these 5 chickens is indicated by the results of the serum hemagglutination inhibition tests given in Table XXII, page 71. In birds 85 and 86 there was no rise in the HI titer. In bird 87 there was a doubtful rise. In 2 birds 88 and 89 the HI titer gave a significant rise in titer but the results were not outstanding. From the results of the HI tests as given in Table XXII it appeared that the concentration of B.P.L. was such that the antigenicity of the virus was altered below a satisfactory level.

Serum neutralization tests were done using embryonating chicken eggs to detect infective virus. The results of these tests are given in Table XXII. None of the 5 birds vaccinated with this vaccine showed a significant difference between the prevaccination and postvaccination neutralizing index. The group combined had an average prevaccination neutralizing index of 1.48 and a postvaccination average of 1.72 with a

difference of 0.24. The results of the neutralization tests are in agreement with the hemagglutination inhibition tests in that, there was not a significant increase in antibodies between the prevaccination and postvaccination sera.

Fifteen days following vaccination the 5 birds received a challenging injection of 0.2 ml. of untreated virus intramuscularly. The result of the virus injection is shown in Table XXI, page 70. Two birds, 85 and 86, died from Newcastle disease, the remaining birds although developing symptoms of the disease, recovered after prolonged illness.

The next step was that of adjusting the concentration of the B.P.L. in a vaccine to a point which would retain a large margin of safety for virus pathogenicity, however, retaining antigenicity. Returning to Table I, page 31, it was determined that as little as 0.025 per cent B.P.L. would destroy the pathogenicity of the virus. Therefore, a vaccine was prepared using 0.025 per cent B.P.L. Eighty-two chickens, all of the same age and source were divided into 2 lots of 43 and 39 respectively. The first lot of 43 birds was divided into 4 groups of 13, 10, 10 and 10 birds and received 0.5, 1.0, 1.5 and 2 ml. of the vaccine respectively. The second lot of 23 birds did not receive the vaccine.

Just prior to vaccinating the chickens, all birds were bled; their sera were held in a frozen state to await the collection of postvaccination sera.

The vaccinated chickens were observed daily for 15 days and none gave evidence of illness. On the sixteenth day following vaccination the test birds (those receiving the vaccine) were again bled and the sera were separated from the clots and stored in a frozen state.

A challenging injection of 0.2 ml. of virus (LD50 = 2 x $10^{9.5}$) was given on the sixteenth day to both the vaccinated and control birds. Again none of the vaccinated chickens gave evidence of disease while the non-vaccinated group began to show evidence of Newcastle disease on the fourth day following the challenge injection of virus. The final results of virus injection in the non-vaccinated birds are given in Table IX. page 58. These birds showed dullness which rapidly developed into a marked depression accompanied by progressive weakness. Cyanosis of the comb and diarrhea were the prominent symptoms. Figure 1, page 53, illustrates the vaccinated and non-vaccinated chickens on the seventh day following the challenging injection of live virus. Deaths in the non-vaccinated birds began on the fifth day following virus injection and by the ninth day 38 of the 39 birds (97.4%) had become ill and died. Only one control bird did not develop Newcastle disease. The disease was fatal to 33 of the 39 (84.6%) chickens.

Hemagglutination inhibition and neutralization tests were done on the paired sera from the vaccinated chickens. The results of HI tests on the 4 groups of vaccinated chickens are given in Tables X through XIII, pages 59-62. Considering the

Figure 1. Chickens vaccinated with Newcastle disease vaccine (0.025% B.P.L.) (left) and non-vaccinated chickens (right) on the seventh day after challenging with virulent Newcastle disease virus.

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results, as given in these 4 tables collectively, there was no evidence that the injection of 0.5, 1.0, 1.5 or 2 ml. of vaccine produced different results. Of the 43 chickens receiving the vaccine, all showed a substantial increase in HI titer when the prevaccination and postvaccination sera were compared. The range of this increase was from 10 to 80 (bird 4011) and 5 to 1600 (bird 4009). The average prevaccination HI titer was 10 and the postvaccination 320.

The results of the serum neutralization test in embryonating chicken eggs on the same paired sera are shown in Tables XVI through XIX, pages 65-68. Only representative samples of serum, chosen at random, were tested for neutralizing antibodies. Considering the results given in these 4 tables collectively, again in every instance there was a marked increase in the antibody titer as seen in the prevaccination and postvaccination indexes. The average prevaccination serum neutralizing index of the 4 groups was 1.19. The average postvaccination index was 4.08. The latter being approximately 4 times greater than the former. Indexes of the prechallenge sera are above those recommended for the diagnosis of Newcastle disease by Osteen and Anderson (1948).

The hemagglutination inhibition tests were done on blood sera from 23 of the non-vaccinated chickens. The results of these tests are given in Table XIV, page 63. Briefly, only one chicken's (189) serum showed a 1:40 HI titer. Referring to Table IX, page 58, it will be seen that, for unknown

reasons, this bird was the only one of 39 birds in the controls that did not show evidence of disease following challenging with live virus. Whether or not the existing 1:40 HI titer in chicken 189 was responsible for its protection against Newcastle disease virus is unknown.

In Table XX, page 69, the HI serum titers of the 4 groups of vaccinated chickens are summarized as well as the titers tested at 2 and 3 months after challenging with untreated virus. The sera were from those birds withstanding the bleeding procedures several times. The majority of sera show a marked increase in HI antibody titer, as would be expected.

To demonstrate that a vaccine containing 0.025 per cent B.P.L. and Newcastle disease virus in allantoic fluid was incapable of producing Newcastle disease, 20 ml. of the vaccine was injected intraperitoneally into a chicken. The chicken receiving this massive dose of vaccine was from the same source as all other chickens in this work. Injection of this amount of Newcastle disease virus vaccine did not produce illness in the bird. This amount of vaccine (20 ml.) represents 10 times the amount of virus that was used in the largest vaccine dose in this study.

Chicken eggs may contain Salmonella pullorum in various numbers. It, therefore, seemed advisable to determine the effect of various concentrations of B.P.L. upon the bacterial cell. Table IV, page 37, gives the results of testing various concentrations of B.P.L. on 2,000,000,000 Salmonella pullorum

organisms contained in 10 ml. of Newcastle disease infected allantoic fluid. It was found that 0.025 and 0.05 per cent B.P.L. did not destroy the organisms. A 0.1 per cent concentration of B.P.L. was found to destroy the bacterial cells in the allantoic fluid. This concentration of B.P.L., however, has been shown to destroy the antigenicity of the Newcastle disease virus.

A vaccine containing 0.025 per cent B.P.L., Newcastle disease virus and *Salmonella pullorum* was made and 0.5 ml. of this mixture was injected into 10 chickens. Sera from these 10 birds all showed an increase in the HI antibody titer (Table XV, page 64) for Newcastle disease virus but failed to demonstrate blood agglutinins to *Salmonella pullorum*. All 10 chickens were destroyed at the end of this experiment and no lesion of Newcastle disease or pullorum infection could be determined.

The effects of increasing concentration of B.P.L. on the viruses of infectious laryngotracheitis and bronchitis are given in Tables VI and V, pages 42 and 40. With both of these viruses which are infectious to the chicken, 0.025 per cent B.P.L. was found to destroy the infectiveness of the agent as indicated by the results of testing in embryonating chicken eggs.

TABLE VIII

RESULTS OF IMMUNITY CHALLENGED OF VACCINATED CHICKENS
(0.025% B.P.L. VACCINE) 0.2 ML. VIRUS INTRAMUSCULARLY

Number of Birds	Amount of Vaccine (ml.)	Date Vaccinated	Date Challenged	Result*
13	0.5	10-26-54	11-12-54	-
10	1.0	10-26-54	11-12-54	-
10	1.5	10-26-54	11-12-54	-
10	2.0	10-26-54	11-12-54	-

* - No reaction

TABLE IX

RESULTS OF VIRUS CHALLENGE OF NON-VACCINATED CONTROL CHICKENS 0.2 ML. VIRUS INTRAMUSCULARY

Birds Number	Symptoms Days After Challenged	Death Days After Challenged	Birds Number	Symptoms Days After Challenged	Death Days After Challenged
164	4	5	195	4	9
4083	4	5	4077	4	9
173	4	5	4094	4	9
4082	4	5	4007	4	9
4096	4	5	152	4	9
167	4	5	172	4	Killed
166	4	5	4097	4	Killed
175	4	5	4081	4	Killed
4078	4	5	4089	4	Killed
179	4	5	170	4	Killed
4085	4	5	4088	4	Recovered
171	4	6	182	4	Recovered
4095	4	6	4084	4	Recovered
186	4	6	4091	4	Recovered
184	4	6	190	4	Recovered
4090	4	6	189	None	0
4098	4	7			
193	4	7			
169	4	7			
4086	4	7			
4100	4	7			
151	4	7			
4092	4	7			

TABLE X

RESULTS OF HEMAGGLUTINATION INHIBITION TESTS ON SERA FROM VACCINATED CHICKENS GROUP 1 (VACCINE 0.5 ML.)

Number Chicken Serum	Virus Dilution									Serum HI Titer
	Und.	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	
102A	+	+	+	+	+	+	-	-	-	10
102B	+	-	-	-	-	-	-	-	-	320
128A	+	+	+	+	+	+	-	-	-	10
128B	+	-	-	-	-	-	-	-	-	320
154A	+	+	+	+	+	-	-	-	-	20
154B	+	-	-	-	-	-	-	-	-	320
4016A	+	+	+	+	+	+	-	-	-	10
4016B	+	-	-	-	-	-	-	-	-	320
159A	+	+	+	+	+	+	+	-	-	5
159B	+	-	-	-	-	-	-	-	-	320
156A	+	+	+	+	-	-	-	-	-	40
156B	+	+	-	-	-	-	-	-	-	160
157A	+	+	+	+	-	-	-	-	-	40
157B	+	-	-	-	-	-	-	-	-	320
143A	+	+	+	+	+	+	-	-	-	10
143B	-	-	-	-	-	-	-	-	-	1600
126A	+	+	+	+	+	+	+	-	-	5
126B	+	-	-	-	-	-	-	-	-	320
101A	+	+	+	+	+	+	-	-	-	10
101B	-	-	-	-	-	-	-	-	-	1600
129A	+	+	+	+	+	+	-	-	-	10
129B	+	-	-	-	-	-	-	-	-	320
PA	+	+	+	+	+	-	-	-	-	20
PB	+	-	-	-	-	-	-	-	-	320
139A	+	+	+	+	+	+	-	-	-	10
139B	+	-	-	-	-	-	-	-	-	320

A = Prevaccinated serum + = Hemagglutination

B = Postvaccinated serum - No hemagglutination

Virus control HA titer 1:320

TABLE XI

RESULTS OF HEMAGGLUTINATION INHIBTION TESTS ON SERA FROM VACCINATED CHICKENS GROUP 2 (VACCINE 1.0 ML.)

Number Chicken Serum	Virus Dilutions									Serum HI Titer
	Und.	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	
160A	+	+	+	+	+	+	+	-	-	5
160B	-	-	-	-	-	-	-	-	-	1600
153A	+	+	+	+	-	-	-	-	-	40
153B	-	-	-	-	-	-	-	-	-	1600
163A	+	+	+	+	+	+	+	-	-	5
163B	+	+	-	-	-	-	-	-	-	160
4011A	+	+	+	+	+	+	-	-	-	10
4011B	+	+	+	-	-	-	-	-	-	80
165A	+	+	+	+	+	-	-	-	-	20
165B	+	-	-	-	-	-	-	-	-	320
168A	+	+	+	+	+	+	-	-	-	10
168B	+	+	-	-	-	-	-	-	--	160
140A	+	+	+	+	+	-	-	-	-	20
140B	+	-	-	-	-	-	-	-	-	320
131A	+	+	+	+	-	-	-	-	-	40
131B	+	-	-	-	-	-	-	-	-	320
135A	+	+	+	+	+	+	+	-	-	5
135B	+	-	-	-	-	-	-	-	-	320
4014A	+	+	+	+	+	+	-	-	-	10
4014B	+	-	-	-	-	-	-	-	-	320

A = Prevaccinated serum
B = Postvaccinated serum
\+ Hemagglutination
\- No hemagglutination
Virus control HA titer 1:320

TABLE XII

RESULTS OF HEMAGGLUTINATION INHIBITION TESTS ON SERA FROM VACCINATED CHICKENS GROUP 3 (VACCINE 1.5 ML.)

Number Chicken Serum	Virus Dilutions Und.	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	Serum HI Titer
147A	+	+	+	+	+	+	+	-	-	5
147B	+	+	+	-	-	-	-	-	-	180
138A	+	+	+	+	+	+	+	-	-	5
138B	-	-	-	-	-	-	-	-	-	1600
127A	+	+	+	+	+	+	-	-	-	10
127B	+	-	-	-	-	-	-	-	-	320
4010A	+	+	+	+	+	-	-	-	-	20
4010B										320
130A	+	+	+	+	+	-	-	-	-	20
130B	+	-	-	-	-	-	-	-	-	320
162A	+	+	+	+	+	+	-	-	-	10
162B	-	-	-	-	-	-	-	-	-	1600
4009A	+	+	+	+	+	+	+	-	-	5
4009B	-	-	-	-	-	-	-	-	-	1600
148A	+	+	+	+	+	+	+	-	-	5
148B	+	-	-	-	-	-	-	-	-	320
187A	+	+	+	+	-	-	-	-	-	40
187B	+	-	-	-	-	-	-	-	-	320
149A	+	+	+	+	+	+	-	-	-	10
149B	+	-	-	-	-	-	-	-	-	320

A = Prevaccinated serum
B = Postvaccinated serum
+ Hemagglutination
- No hemagglutination
Virus control HA titer 1:320

TABLE XIII

RESULTS OF HEMAGGLUTINATION INHIBITION TESTS ON SERA FROM VACCINATED CHICKENS GROUP 4 (VACCINE 2.0 ML.)

Number Chicken Serum	Virus Dilution									Serum HI Titer
	Und.	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	
146A	+	+	+	+	+	+	+	-	-	5
146B	+	-	-	-	-	-	-	-	-	320
4093A	+	+	+	+	-	-	-	-	-	40
4093B	+	-	-	-	-	-	-	-	-	320
4099A	+	+	+	+	+	+	-	-	-	10
4099B	+	-	-	-	-	-	-	-	-	320
4012A	+	+	+	+	+	+	-	-	-	10
4012B	+	-	-	-	-	-	-	-	-	320
145A	+	+	+	+	-	-	-	-	-	40
145B	-	-	-	-	-	-	-	-	-	1600
181A	+	+	+	+	+	-	-	-	-	20
181B	-	-	-	-	-	-	-	-	-	1600
133A	+	+	+	+	+	+	-	-	-	10
133B	-	-	-	-	-	-	-	-	-	1600
141A	+	+	+	+	+	-	-	-	-	20
141B	-	-	-	-	-	-	-	-	-	1600
161A	+	+	+	+	+	+	-	-	-	10
161B	+	-	-	-	-	-	-	-	-	320
158A	+	+	+	+	+	-	-	-	-	20
158B	+	-	-	-	-	-	-	-	-	320

A = Prevaccinated serum
B = Postvaccinated serum
+ Hemagglutination
- No hemagglutination
Virus control HA titer 1:320

TABLE XIV

RESULTS OF HEMAGGLUTINATION INHIBITION TESTS ON SERA FROM NON-VACCINATED CONTROL CHICKENS

Chicken Serum Number	Virus Dilution Und.	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	Virus HI Titer
4084	+	+	+	+	+	+	-	-	-	10
4089	+	+	+	+	+	-	-	-	-	20
4091	+	+	+	+	+	+	+	-	-	5
4080	+	+	+	+	+	-	-	-	-	20
189	+	+	+	+	-	-	-	-	-	40
186	+	+	+	+	+	+	+	-	-	5
4097	+	+	+	+	+	+	-	-	-	10
151	+	+	+	+	+	+	+	-	-	5
4083	+	+	+	+	+	-	-	-	-	20
195	+	+	+	+	+	+	-	-	-	10
4077	+	+	+	+	+	-	-	-	-	20
179	+	+	+	+	+	+	+	-	-	5
194	+	+	+	+	+	+	-	-	-	10
4007	+	+	+	+	+	-	-	-	-	20
170	+	+	+	+	+	+	-	-	-	10
4086	+	+	+	+	+	+	+	-	-	5
4088	+	+	+	+	+	+	-	-	-	10
4096	+	+	+	+	+	+	+	-	-	5
169	+	+	+	+	+	+	-	-	-	10
4090	+	+	+	+	+	+	-	-	-	10
164	+	+	+	+	+	+	-	-	-	10
182	+	+	+	+	+	+	+	-	-	5
172	+	+	+	+	+	+	+	-	-	5

+ Hemagglutination
- No hemagglutination
Virus control HA titer 1:320

TABLE XV

RESULTS OF HEMAGGLUTINATION INHIBITION TESTS ON SERA FROM CHICKENS VACCINATED WITH 0.5 ML. NDV-0.025% B.P.L. VACCINE CONTAINING <u>SALMONELLA</u> <u>PULLORUM</u>

Number Chicken Serum	Und.	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	Serum HI Titer
	Virus Dilution									
90A	+	+	+	+	+	+	+	-	-	5
90B	+	-	-	-	-	-	-	-	-	320
91A	+	+	+	+	+	+	+	-	-	5
91B	+	+	+	+	-	-	-	-	-	40
93A	+	+	+	+	+	-	-	-	-	20
93B	-	-	-	-	-	-	-	-	-	1600
94A	+	+	+	+	+	+	-	-	-	10
94B	+	+	-	-	-	-	-	-	-	160
95A	+	+	+	+	+	+	+	-	-	5
95B	+	-	-	-	-	-	-	-	-	320
96A	+	+	+	+	+	+	-	-	-	10
96B	+	+	-	-	-	-	-	-	-	160
97A	+	+	+	+	+	+	+	-	-	5
97B	+	-	-	-	-	-	-	-	-	320
98A	+	+	+	+	+	+	+	-	-	5
98B	+	-	-	-	-	-	-	-	-	320
99A	+	+	+	+	+	+	+	-	-	5
99B	+	+	+	-	-	-	-	-	-	80
100A	+	+	+	+	+	+	+	-	-	5
100B	+	-	-	-	-	-	--	-	-	320

A = Prevaccinated serum
B = Postvaccinated serum
+ Hemagglutination
- No Hemagglutination
Virus control HA titer 1:320

TABLE XVI

RESULTS OF SERUM NEUTRALIZATION TESTS IN EGGS FROM VACCINATED CHICKENS GROUP 1 (VACCINE 0.5 ML.)

		Virus Dilution							Serum	
Chicken Serum Number	Virus Titer	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Titer	Neutralizing Index
102A #	9.62	-	-	-	5/5	4/5	2/5	0/5	7.64	1.98
102B	9.62	-	5/5	5/5	4/5	0/5	-	-	6.37	3.25
128A	9.62	-	-	-	5/5	5/5	1/4	0/5	7.66	1.96
128B	9.62	5/5	2/5	1/5	0/5	-	-	-	3.66	5.96
154A	9.48	-	-	-	5/5	5/5	3/5	1/5	8.32	1.16
154B	9.48	5/5	5/5	4/5	0/5	-	-	-	5.37	4.11
4016A	9.48	-	-	-	5/5	5/5	3/5	0/5	8.16	1.52
4016B	9.48	5/5	5/5	1/5	0/5	-	-	-	5.48	4.0
159A	9.48	-	-	-	5/5	5/5	5/5	0/5	8.5	0.98
159B	9.48	5/5	5/5	5/5	3/5	-	-	-	6.16	3.32
156A	9.62	-	-	-	5/5	5/5	4/4	0/5	8.5	2.12
156B	9.62	-	-	5/5	5/5	3/4	0/5	-	7.33	2.29
157A	9.45	-	-	-	5/5	4/5	2/4	0/4	7.78	1.67
157B	9.45	4/4	5/5	5/5	3/5	-	-	-	6.16	3.29
143A	9.48	-	-	-	5/5	5/5	4/5	2/5	8.63	0.85
143B	9.48	5/5	3/5	0/5	0/5	-	-	-	4.66	4.82
126A	8.49	-	-	-	5/5	3/4	0/4	0/5	7.33	1.16
126B	8.49	5/5	4/4	3/5	1/5	-	-	-	5.32	3.17

Average prevacclnation Neutralizing index 1.37
Average postvaccination Neutralizing index 3.80

A Prevaccinated serum
B Postvaccinated serum

$\frac{\text{Numerator number dead}}{\text{Denominator number injected}}$

TABLE XVII

RESULTS OF SERUM NEUTRALIZATION TESTS IN EGGS FROM VACCINATED CHICKENS GROUP 2 (VACCINE 1 ML.)

		Virus Dilution							Serum	
Chicken Serum Number	Virus Titer	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Titer	Neutralizing Index
160A #	9.48	-	-	-	5/5	5/5	2/5	0/5	7.83	1.65
160B	9.48	3/5	2/5	0/5	0/5	-	-	-	3.5	5.9
153A	9.48	-	-	-	5/5	3/5	2/5	0/5	7.48	2.0
153B	9.48	4.5	3/5	2/5	0/5	-	-	-	4.33	5.15
163A	9.45	-	-	-	5/5	5/5	5/5	2/5	8.83	0.63
163B	9.45	5/5	5/5	3/5	2/4	-	-	-	5.56	3.89
4011A	8.16	-	-	-	5/5	5/5	4/5	0/5	7.62	0.54
4011B	8.16	5/5	5/5	5/5	3/5	-	-	-	6.16	2.0
165A	8.16	-	-	-	5/5	5/5	0/5	0/5	7.5	0.66
165B	8.16	5/5	5/5	2/5	1/5	-	-	-	5.0	3.16
168A	8.16	-	-	-	5/5	5/5	0/5	0/5	7.5	0.66
168B	8.16	5/5	5/5	3/5	0/5	-	-	-	5.16	3.0

Average prevaccination Neutralizing index 1.023
Average postvaccination Neutralizing index 3.85

\# A Prevaccinated serum
B Postvaccinated serum

$\frac{\text{Numerator number dead}}{\text{Denominator number injected}}$

TABLE XVIII

RESULTS OF SERUM NEUTRALIZATION TESTS IN EGGS FROM VACCINATED CHICKENS GROUP 3 (VACCINE 1.5 ML.)

Chicken Serum Number	Virus Titer	Virus Dilution 10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Serum Titer	Neutralizing Index
147A #	8.49	-	-	-	5/5	4/5	2/4	0/5	7.78	0.71
147B	8.49	5/5	5/5	5/5	3/5	-	--	-	6.16	2.33
138A	8.49	-	-	-	5/5	3/5	0/5	0/5	7.16	1.33
138B	8.49	3/5	1/5	0/5	0/5	-	-	-	3.32	5.17
127A	8.49	-	-	-	5/5	5/5	2/5	0/5	7.83	0.66
127B	8.49	4/4	5/5	5/5	1/5	-	-	-	5.62	2.87
4010A	9.83	-	-	-	5/5	5/5	5/5	0/5	8.50	1.33
4010B	9.50	5/5	5/5	4/5	2/5	-	-	-	5.64	3.86
130A	9.48	-	-	-	5/5	5/5	5/5	0/5	8.50	0.98
130B	9.48	5/5	5/5	3/5	0/5	-	-	-	5.16	4.32
162A	8.49	-	-	-	5/5	3/5	2/5	0/5	7.48	1.01
162B	8.49	5/5	4/5	1/5	0/5	-	-	-	4.49	4.00

Average prevaccination Neutralizing index 1.003
Average postvaccination Neutralizing index 3.758

\# A Prevaccinated serum
B Postvaccinated serum

$\frac{\text{Numerator number dead}}{\text{Denominator number injected}}$

TABLE XIX

RESULTS OF SERUM NEUTRALIZATION TESTS IN EGGS FROM VACCINATED CHICKENS GROUP 4 (VACCINE 2.0 ML.)

		Virus Dilution							Serum	
Chicken Serum Number	Virus Titer	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Titer	Neutralizing Index
146A #	9.83	-	-	-	5/5	5/5	5/5	3/5	9.16	0.67
146B	9.83	5/5	4/5	0/5	0/5	-	-	-	4.83	5.45
4093A	9.83	-	-	-	5/5	5/5	5/5	0/5	8.50	1.83
4093B	9.83	5/5	3/4	0/5	0/5	-	-	-	4.33	5.50
4099A	9.48	-	-	-	5/5	5/5	2/5	0/5	7.83	1.65
4099B	9.48	5/5	5/5	1/5	0/5	-	-	-	4.83	3.56
4012A	8.49	-	-	-	5/5	5/5	0/5	0/5	7.50	0.99
4012B	8.49	5/5	5/5	2/5	0/5	-	-	-	4.83	3.56
145A	9.48	-	-	-	5/5	5/5	2/5	1/5	8.0	1.48
145B	9.48	4/5	2/5	0/5	0/5	-	-	-	3.78	5.70
181A	9.48	-	-	-	5/5	5/5	2/5	0/5	7.83	1.65
181B	9.45	3/4	2/5	0/5	0/5	-	-	-	3.60	5.85

Average prevaccination Neutralizing index 1.378
Average postvaccination Neutralizing index 4.936

\# A Prevaccinated serum
B Postvaccinated serum

Numerator number dead / Denominator number injected

TABLE XX

RESULTS OF HEMAGGLUTINATION INHIBITION TESTS ON SERA FROM VACCINATED BIRDS

Bird Number	ml. Vaccine Received	Prevaccination HI Titer	Postvaccination HI Titer	Postchallenged HI Titer* 2 months	3 months
102	0.5	10	320	1280	-
139	0.5	10	320	-	1280
128	0.5	10	320	2560	-
101	0.5	10	1600	-	2560
129	0.5	10	320	-	2560
154	0.5	20	320	-	-
4016	0.5	10	320	-	2560
159	0.5	5	320	1280	-
156	0.5	40	160	-	1280
157	0.5	40	320	-	2560
143	0.5	10	1600	-	2560
126	0.5	5	320	2560	-
P	0.5	20	320	-	2560
168	1.0	10	160	2560	-
4011	1.0	10	80	2560	-
140	1.0	20	320	2560	-
4014	1.0	10	320	-	2560
135	1.0	5	320	2560	-
131	1.0	40	320	640	-
165	1.0	20	320	-	2560
163	1.0	5	160	640	-
160	1.0	5	1600	-	1280
153	1.0	40	1600	-	1280
4009	1.5	5	1600	2560	-
147	1.5	5	180	2560	-
148	1.5	5	320	-	-
187	1.5	40	320	2560	-
138	1.5	5	1600	-	2560
149	1.5	10	320	-	2560
127	1.5	10	320	2560	-
4010	1.5	20	320	-	2560
130	1.5	20	320	-	2560
162	1.5	10	1600	-	2560
133	2.0	10	320	-	2560
146	2.0	5	320	640	-
4093	2.0	40	320	2560	-
4099	2.0	10	320	1280	-
4012	2.0	10	320	2560	-
141	2.0	20	1600	2560	-
145	2.0	40	1600	-	2560
161	2.0	10	320	-	2560
181	2.0	20	1600	--	-
158	2.0	20	320	-	-

* Sera diluted 1:40
- Hemagglutination-inhibition test not done

TABLE XXI

IMMUNITY CHALLENGED OF VACCINATED CHICKENS (0.5 ML. OF 1% B.P.L. VACCINE) 0.2 ML. VIRUS INTRAMUSCULARLY

Birds Number	Date Vaccinated	Date Challenged	Result
85	2-2-55	2-17-55	Died on 2-22-55, Newcastle disease
86	2-2-55	2-17-55	Died on 2-22-55, Newcastle disease
87	2-2-55	2-17-55	Off feed, depression, sneezing and gasping, no nervous symptoms. Recovered.
88	2-2-55	2-17-55	Symptoms of Newcastle disease as above and recovered.
89	2-2-55	2-17-55	Symptoms of Newcastle disease as above and recovered.

TABLE XXII

RESULTS OF HEMAGGLUTINATION INHIBITION TESTS ON SERA FROM VACCINATED CHICKENS 0.1% B.P.L. VACCINE (AMOUNT 0.5 ML.)

Number Chicken Serum	Virus Dilution									Serum HI Titer
	Und.	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	
85A	+	+	+	+	+	-	-	-	-	20
85B	+	+	+	+	+	-	-	-	-	20
86A	+	+	+	-	-	-	-	-	-	80
86B	+	+	+	-	-	-	-	-	-	80
87A	+	+	+	+	+	-	-	-	-	20
87B	+	+	+	+	-	-	-	-	-	40
88A	+	+	+	+	+	+	-	-	-	10
88B	+	-	-	-	-	-	-	-	-	320
89A	+	+	+	+	+	+	+	-	-	5
89A	+	+	-	-	-	-	-	-	-	160

A = Prevaccinated serum
B = Postvaccinated serum
+ Hemagglutination
- No hemagglutination
Virus control HA titer 1:320

TABLE XXIII

RESULTS OF SERUM NEUTRALIZATION TESTS IN EGGS FROM VACCINATED CHICKENS VACCINE CONTAINED 0.1% B.P.L. (AMOUNT 0.5 ML.)

		Virus Dilution							Serum	
Chicken Serum Number	Virus Titer	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Titer	Neutral-izing Index
85A #	9.50	-	-	-	5/5	5/5	5/5	3/5	9.16	0.34
85B	8.83	-	-	-	5/5	5/5	1/5	0/5	7.62	1.21
86A	9.50	-	-	-	5/5	5/5	3/5	0/5	8.16	1.34
86B	8.83	-	-	-	5/5	5/5	1/5	0/5	7.62	1.21
87A	9.50	-	-	-	5/5	5/5	2/5	0/5	7.83	1.67
87B	8.83	-	-	-	5/5	5/5	2/5	0/5	7.83	1.00
88A	9.50	-	-	-	5/5	5/5	1/5	0/5	7.62	1.88
88B	9.50	-	-	5/5	3/5	2/5	1/5	0/5	6.66	2.84
89A	9.50	-	-	-	5/5	3/5	0/5	0/5	7.16	2.18
89B	9.50	-	-	4/5	4/4	3/5	0/5	0/5	7.16	2.34

Average prevaccination Neutralizing index 1.48
Average postvaccination Neutralizing index 1.72

\# A Prevaccinated serum
B Postvaccinated serum

$\frac{\text{Numerator number dead}}{\text{Denominator number injected}}$

V. DISCUSSION

The results give evidence that beta-propiolactone is an excellent compound to alter Newcastle disease virus. When 0.025 per cent B.P.L. was used with the virus, the pathogenicity was so altered that infection was not observed. Although the infectivity was destroyed the antigenicity of the virus was retained. Challenging amounts of virus failed to produce disease in chickens vaccinated by the method described. The serological evidence of antibody stimulation was found by both the serum neutralization and hemagglutination inhibition tests. This evidence was marked in every chicken tested although the time between vaccination and challenging virus was comparative short.

Advantages of this vaccine are many: Other virus infections (excluding lymphomatosis), such as infectious bronchitis and laryngotracheitis viruses were destroyed by the concentration of B.P.L. best suited to produce a potent vaccine. Although the number of pullorum organisms used in this experiment was probably in gross excess of that naturally occurring in infected eggs, there was evidence of bactericidal effect upon the organisms. No agglutinins for pullorum were found in the sera of those birds receiving the vaccine containing the organisms.

There would be small likelihood of dissemination of the Newcastle disease virus in a vaccine of this type because of the absence of infectivity. Evidence supporting this is the fact that the very sensitive embryonating eggs did not show existence of living virus; and when a massive dose was administered to a chicken, no symptoms of illness resulted. A properly made vaccine would be incapable of "field break."

A 0.025 per cent concentration of B.P.L. destroyed the infectivity of infectious bronchitis and laryngotracheitis viruses. It is, therefore, possible to incorporate these viruses, providing their antigenicity is not altered, into a vaccine. If effective, a triple virus vaccine of this nature would be very desirable and convenient.

There remain other questions to be answered before B.P.L. can be used extensively with Newcastle disease. For instance, what effect does B.P.L. have on other strains of Newcastle, lymphomatosis, infectious bronchitis and laryngotracheitis viruses? What is the duration of solid resistance to the disease considering the volume of vaccine used? However, studies of pathogenicity and antigenicity, showed that the use of B.P.L. treated Newcastle disease virus is basically sound.

VI. SUMMARY

1. The virucidal effect of beta-propiolactone (B.P.L.) upon Newcastle disease, infectious bronchitis and laryngotracheitis viruses was determined.

2. It was found that 0.025 per cent B.P.L. completely destroyed the infectious properties of Newcastle disease virus but did not destroy the antigenicity.

3. A vaccine made by adding 0.025 per cent B.P.L. to Newcastle virus was shown to completely protect chickens against a challenging injection of live virus. Control chickens not receiving the vaccine but challenged with live virus showed typical symptoms of Newcastle disease and the majority of birds died from the infection.

4. Paired sera collected from the chickens before and after vaccination showed a marked rise in neutralizing and hemagglutination inhibition antibodies.

5. The same concentration of B.P.L. (0.025 per cent) as was used to make the Newcastle disease vaccine, was shown to destroy the infectivity of infectious bronchitis and laryngotracheitis viruses.

6. Large numbers of _Salmonella pullorum_ were not destroyed by 0.025 per cent B.P.L. A vaccine made for Newcastle disease and containing _S. pullorum_ was injected into chickens. The chickens did not show evidence of either Newcastle disease or

pullorum infection and at the end of this experiment these birds were sacrificed and no evidence of bacterial infection could be found. The sera from these birds did not give evidence of pullorum agglutinins at the close of the experiment.

7. Advantages of this type of vaccine are discussed.

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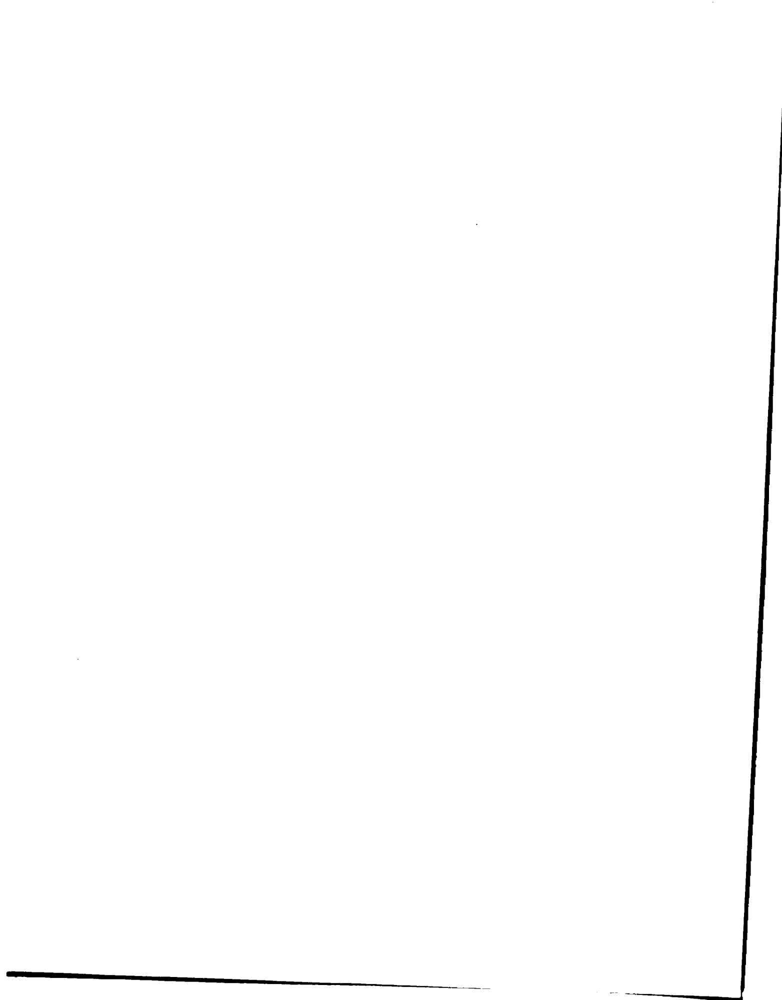

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