A STUDY OF SOME VARIABLES IN THE NEUTRALIZATION TEST AS USED FOR POTENCY TESTING COMMERCIALLY AVAILABLE INFECTIOUS BRONCHITIS VACCINES

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By

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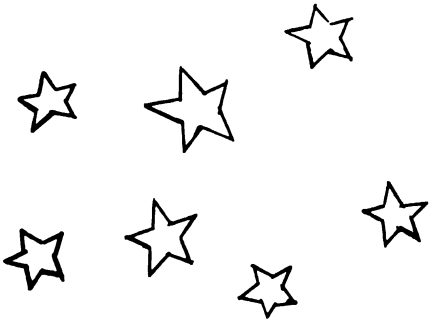

When you reach for the stars
You may not quite get one,
But you won't come up with a handful of mud, either.

Anon.

Dedicated

to

HELEN

and

our sons

for all the evenings they went

without television while

this manuscript was

prepared.

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INTRODUCTION

Infectious bronchitis (IB) is an acute, highly contagious respiratory disease of chickens of great economic importance to the poultry industry. The problems encountered in the prevention and control of this disease are of such magnitude that the Committee on Transmissible Diseases of the United States Livestock Sanitary Association has devoted many years of study to fundamental aspects of the disease.

The introduction of live virus infectious bronchitis vaccines for control of the disease has uncovered new problems associated with the disease. Recognizing the need for standard procedures for testing the efficacy of infectious bronchitis vaccines, the United States Department of Agriculture assigned the author the responsibility for developing, evaluating, and improving procedures to be used by the Veterinary Biologics Licensing and Inspection Sections of the U. S. Department of Agriculture and by the licensed laboratories for the testing and evaluation of these vaccines.

A neutralization test procedure is employed to measure the viral infectivity neutralizing property of antibody and is used by licensed laboratories, state and federal regulatory agencies, diagnostic laboratories, and research workers for assay of immunity produced in chickens by infectious bronchitis virus (IBV), including the IB vaccines.

The reliability of the neutralization test for measuring the degree of immunity stimulated by IB vaccines has been questioned by several investigators (Hofstad, 1959b; Raggi and Lee, 1957; Jungherr, et al., 1956a,b; Raggi and Bankowski, 1956). Reciprocal tests where the relationship between the neutralization index (NI) and the response to intratracheal challenge of chickens vaccinated with various strains of IBV were examined. Cross challenge tests with some strains did not give results that agreed with the neutralization test results. Some strains revealed low heterologous neutralization indices compared to the homologous indices but, on challenge, these chickens were completely resistant to some of the strains used and partially resistant to other strains. Further, with some strains of IBV it appeared that neither the neutralization test nor the intratracheal challenge seemed to provide absolute means for detecting an immune status of chickens against IBV.

The present study is part of the assignment by the Veterinary Biologics Licensing and Inspection Sections of the United States Department of Agriculture and is concerned with: (1) fundamental and applied virology as reflected in the neutralization test, (2) some of the variables in the neutralization test, and (3) interpretation of results when assaying the efficacy of commercially available IB vaccines.

REVIEW OF LITERATURE

Etiological Agent

Infectious bronchitis is caused by the virus, Tarpei pulli. The virus is spherical. The diameter ranges from 65 to 135 mμ (Cunningham, 1957; Hofstad, 1959a; Nazerian, 1960). The disease has been reported in the United States and Canada, and also in England, Japan and the Netherlands (Hofstad, 1959a). It is also known to exist in other parts of the world.

Infectious bronchitis virus grown in the developing chicken embryo causes death or characteristic gross lesions such as stunting and curling of the embryo, thickened amnion, clubbed down, and foci of urate-like material in the kidneys after a few serial passages (Loomis, et al., 1950; Hitchner and White, 1955). Further serial passage in the chicken embryo attenuates IBV by reducing the virulence for chickens but increasing the mortality rate for chicken embryos.

IBV can also be adapted to and grown in various cultures of chicken embryonic cells where cytopathic effects (CPE) are produced (Chomiak, et al., 1958; Spring, 1960; Chang, 1960).

Epidemiology

The incubation period for the naturally occurring disease in chickens is from 18 to 36 hours depending upon the amount and strain of virus, degree of attenuation resulting from serial passage in embryos, and the route of inoculation. Susceptible chickens can be readily infected by aerosol, intranasal, ocular, or intratracheal inoculation of virus-infected allantoic fluid or of tracheal exudate and lung tissue suspensions from infected chickens (Hofstad, 1959a).

Natural spread of the disease requires 36 hours or more whereas artificial infection regularly produces tracheal rales within 18 to 30 hours. Death or recovery usually occurs within 6 to 18 days (Hofstad, 1959a).

The most characteristic signs of infection in chicks are nasal discharge, wet eyes, gasping, rales, and coughing or sneezing. Uncomplicated IB seldom persists for more than a week in the individual chicken but it spreads rapidly throughout the entire flock. With secondary complications the mortality rate may be as much as 25 per cent in chicks under 2 weeks of age, but in chickens over 6 weeks of age mortality is negligible. In older chickens the signs are similar to those in chicks but a nasal discharge is usually not seen. In laying flocks production will decline. Misshapen, rough, and soft-shelled eggs of poor quality may be produced even after return

of the flock to production level equivalent to that attained prior to infection (Hofstad, 1959a). In chicks under two weeks of age, IB may cause permanent damage to the ovary resulting in false, or "internal," layers (Sevoian and Levine, 1957). Under certain conditions, IB may be complicated by chronic respiratory disease. Chicks thus affected may exhibit respiratory signs for several weeks and become weak and stunted.

Findings at Necropsy

Gross findings

The characteristic findings are serous or catarrhal exudate in the trachea and catarrhal or fibrinous inflammation (cloudiness) of the air sacs. Yellowish, caseous plugs may be found in the lower trachea and bronchi of chicks that die. Small areas of pneumonia around the large bronchi may occasionally be present. Chicks up to 3 weeks of age may also have a catarrhal inflammation of the nasal passages and sinuses which results in nasal discharge, wet eyes, and occasionally swollen sinuses. With older chicks this condition is less common. In chickens over two months of age involvement of the upper nasal passages and sinuses is seldom encountered (Hofstad, 1959a).

Microscopic findings

Hofstad (1945a) found a thickening of the mucosa and submucosa of the trachea as the result of edema and diffuse cellular infiltration. There was no interruption of the continuity of the tracheal epithelium and the lumen contained an exudate with few or no cellular elements. Inclusion bodies have not been observed. Some authors indicate that differential diagnosis of IB from other respiratory diseases of chickens may be made on the basis of microscopic changes in the trachea (Jungherr, et al., 1956a,b; Chang et al., 1957).

Experimental microscopic findings

Histopathological studies of the tracheal response to artificial infection of chickens with IBV indicate that this response may vary according to dosage, strain of virus, and other factors (Jungherr, et al., 1956a,b; Chang, et al.,1957; Hofstad, 1958, 1959b).

Jungherr, et al.(1956b) divided this response into three sequential phases:

(1) Acute phase (1 to 3 days) -
markedly thickened mucosa,
cilia lost from epithelium,
zone of massive edema with congestion of capillaries, and
slight cellular infiltration under the epithelium;

(2) Reparative phase (6 to 9 days) -
diminished exudate in lumen,
reduced height of epithelium and tendency to form intraepithelial glands, and
marked infiltration of mononuclear elements in former edematous areas;

(3) Immune phase (12 to 18 days) -
return to normal of a large part of mucosa,
cilia almost completely restored,
regenerated epithelial cells somewhat large,
irregular spacing of glands, and
lymphfollicle-like aggregates at intervals in propria.

Low dosages of IBV decreased the intensity of the acute phase and delayed its onset. Under uncomplicated conditions, IBV produced a predictable pathological response in the tracheal mucosa. The recuperative power of the mucosa was remarkable.

Jungherr, et al. (1956b) and Chang, et al. (1957) indicated that histopathological examination of the trachea is superior to clinical examination as a means of assessing infection. The advantages cited were: (1) objective examination within a few days after exposure of chickens, (2) permanent record, and (3) the need for only a few chickens to be held in isolation until their tracheae are collected for examination.

Diagnosis

Differential diagnosis of IB from other viral respiratory infections of poultry is difficult on the basis of clinical signs and lesions. Inclusion bodies are found in cells of chickens infected with fowl pox and infectious fowl laryngotracheitis but not with IB or Newcastle disease. Embryonating chicken eggs are used for isolation of the virus and for neutralization tests for identification of IBV and its antibody.

Virus can be recovered from chickens infected with IBV as long as respiratory signs are evident, usually up to 21 days after exposure (Fabricant, 1949). Antibody in excess of 100 neutralizing doses can be detected as early as the tenth to fourteenth day after exposure to IBV but quantitative assay should not be attempted earlier than 21 to 28 days after exposure to assure sufficient antibodies for a reliable quantitative assay (Page, 1950; Fabricant, 1951; Cunningham, 1952).

Infectious bronchitis virus, unlike Newcastle disease virus, does not possess the ability to agglutinate chicken red blood cells (Hofstad, 1945b) but when it is modified with trypsin, hemagglutination occurs (Corbo and Cunningham, 1959; Muldoon, 1960). Inhibition of hemagglutination by anti-IB serum has not been demonstrated. One means of differentiation of IB from Newcastle disease is by the inhibition of hemagglutination by anti-Newcastle disease serum.

Control Measures

Infectious bronchitis is best prevented by isolation of the flock along with sound management practices such as adding only day-old chicks for replacement stock and rearing them in isolation from the rest of the flock, control of visitors to the poultry house area, and adequate quarantine procedures by the caretaker. In spite of these precautions IB may occur,

particularly in the concentrated areas of poultry population. Immunization for control of the disease is then necessary.

One method for control of IB is the vaccination of chicks with live virus IB vaccine administered by intranasal or ocular drop, by incorporation in the drinking water, by aerosol, or by dust. A second vaccination is recommended eight to ten weeks later. The last three methods are techniques for mass vaccination that eliminate individual handling of large numbers of chickens but may leave susceptible individuals after vaccination.

The vaccines presently available contain live strains of IBV that have been modified by serial passage in chicken embryos to reduce the pathogenicity of the virus but to retain its immunogenicity for chickens (Hofstad, 1959a). The degree of immunity produced against IB following the use of commercially available vaccines is variable with unsatisfactory antibody levels being produced in some instances (Raggi and Lee, 1958).

Some of the factors that may be deleterious to an effective immunization program are as follows:

(1) undesirable pathogenic and immunogenic properties of the strain of virus used to produce the vaccine,

(2) virus titer of the vaccine too low to stimulate adequate protection against infection,

(3) unsatisfactory stabilizing agent used in the vaccine to protect the vaccine from deterioration,

(4) improper refrigeration of the vaccine at any period from the time of production to the time of use,

(5) poor flock management resulting in parasitized and diseased chickens,

(6) variation in parental immunity between the individual chicks at time of vaccination, and

(7) improper dosage and administration of the vaccine.

The poultryman and the manufacturer of the vaccine each have their areas of responsibility for assuring that all chickens receive the correct amount of an efficacious vaccine at the proper time for adequate immunization against IB.

Live virus IB vaccine has also been combined with live virus Newcastle disease vaccine as an added convenience for administration. These two vaccines are officially designated and licensed by the United States Department of Agriculture as Infectious Bronchitis Vaccine, live virus, and the combined product as Newcastle Disease Vaccine, B_1 type, and Infectious Bronchitis Vaccine, live virus.

The first license for the manufacture of Infectious Bronchitis Vaccine, live virus, for interstate distribution and use was issued by the U. S. Department of Agriculture in 1953. Since then the number of doses produced in the United States

has increased steadily and in 1959 over 1 1/2 billion doses were produced (Table 1; figure 1).

Vaccines containing live virus generally produce a reaction of varying degree that indicates a specific immunological response by the recipient. For example, when chickens are vaccinated against IB with the modified live virus vaccines, there is usually a mild respiratory reaction such as sneezing and light rales observable sometime between the third and ninth day after vaccination. The degree of reaction produced by different strains of IBV varies from mild to severe.

It should be emphasized that while these vaccines are safe to use under ideal conditions, they may initiate a focus of inapparent IB capable of infecting susceptible chicks in a flock. Undesirable results may occur (1) in chicks with passive antibodies from their dams or (2) in laying flocks in high production. Chicks hatched from eggs laid by immune hens possess varying degrees of parental immunity that may interfere with immunization if they are vaccinated at an early age (Markham, 1959). Such chicks may be susceptible by the time they reach maturity.

As with many new products, there is a need for constant re-evaluation and development of test procedures that will accurately assess the efficacy of the various live virus strains of IBV used in vaccines today.

TABLE 1

Doses of infectious bronchitis vaccines produced January 1, 1955 to December 31, 1959.

Doses produced *		
Calendar year	Infectious Bronchitis Vaccine	Combined Newcastle-Bronchitis Vaccine
1955	284,220,600	270,264,500
1956	341,561,000	512,081,500
1957	342,787,350	633,177,700
1958	385,893,750	813,480,500
1959	400,617,400	1,167,386,080

* U. S. Department of Agriculture, Agricultural Research Service, Animal Inspection and Quarantine Division,

Biological Products Notice No. 26, dated 2-20-56.
" " " " 38, " 2-27-57.
" " " " 50, " 3-4-58.
" " " " 62, " 2-27-59.
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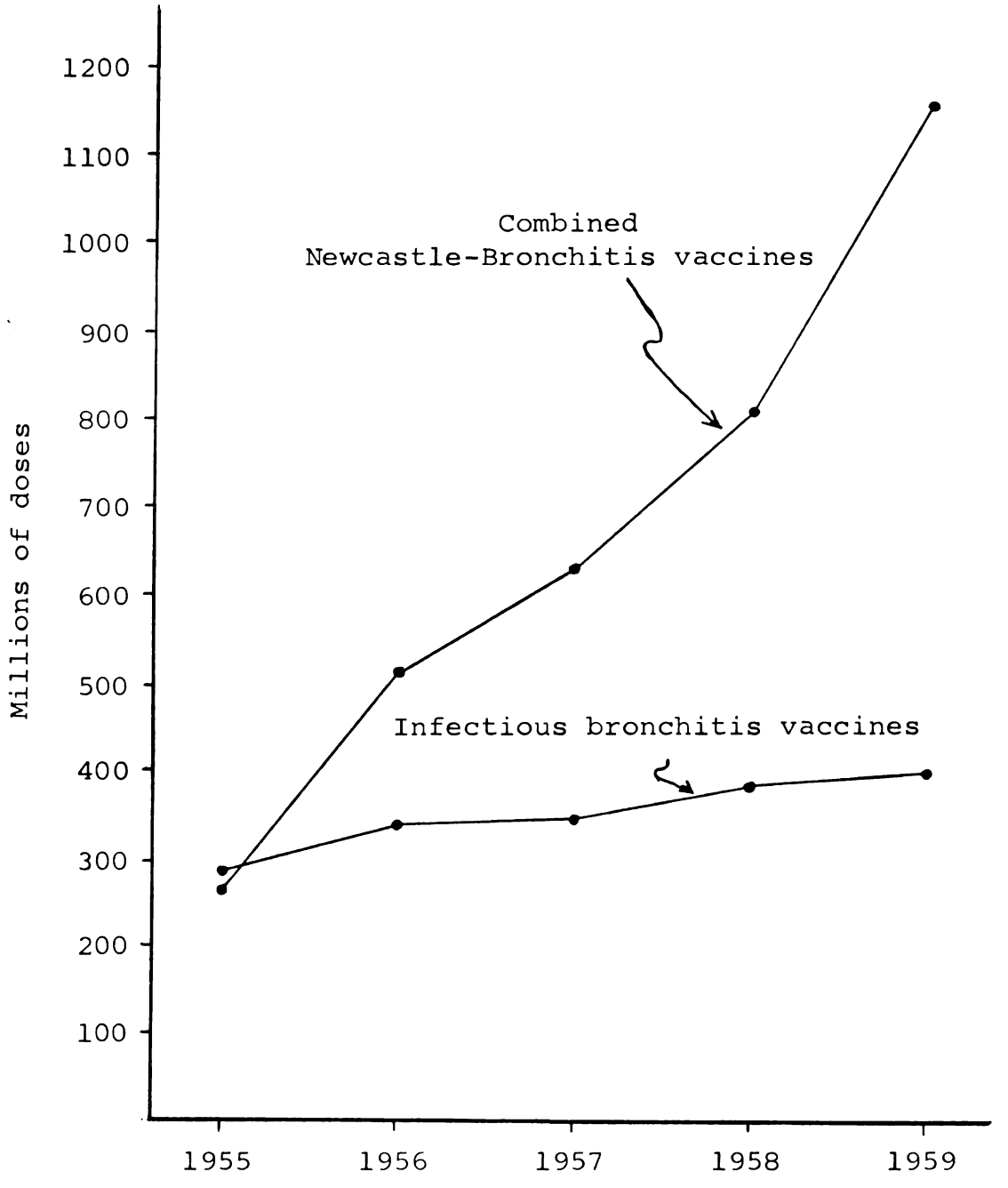

Figure 1. Doses of infectious bronchitis vaccines produced from January 1, 1955, to December 31, 1959.

Properties of Viral Antigen-Antibody Reactions

Campbell and Garvey (1960) reviewed the three generally accepted concepts of antibody formation and added a possible fourth:

(1) Ehrlich's classical "side-chain" theory which is now identified with adaptive or inductive enzyme mechanisms. This idea demands pre-existing templates already functioning in the absence of antigen. It is assumed that antibody is a normal body constituent and that production to detectable levels is stimulated by injection or exposure to antigen;

(2) The template theory postulates that an antigen template must be present for every antibody molecule formed;

(3) The mutation theory which suggests changes involving deoxyribonucleic acid (DNA) and ribonucleic acid (RNA); and

(4) Antibodies may be produced by some combination of the mechanisms postulated in the first three concepts.

Various tests have been developed to indicate the presence or absence of antibodies by the use of antigens in specific antigen-antibody reactions. These reactions are the most sensitive and specific tools for determining antigenic relationships of viruses and their specific antibody. In virology

the basic procedures used for this study are: (1) the complement-fixation test, (2) the hemagglutination-inhibition test, (3) the flocculation (agglutination or precipitation) test, and (4) the neutralization test. Fluorescent antibody techniques are being evaluated for study of antigen-antibody reactions.

Specificity of the reaction between a virus and its antibody is an essential requirement for all these tests. Unfortunately, serological tests are seldom performed with purified antigens or antibodies. They use the rather unpredictable menstruum of serum, the many components of which may neutralize a virus and produce grossly misleading results (Ginsberg and Horsfall, 1949; Wedgwood, et al., 1956; Klein, 1958; Ginsberg, 1960). Sensitivity of the antigen for detection of the homogeneity or heterogeneity of the antibody must also be considered.

Markham (1955) reported that the conditions under which Newcastle disease and infectious bronchitis antiserums are stored and the length of time they are held have an important influence on the extent to which these various non-specific serum factors may contribute to the real and apparent neutralizing capacity of serum. He reported the loss of 0.5 to 1.5 logs of neutralizing capacity following heat inactivation.

Howitt (1950) reported that heating to 56 C for 30 minutes destroyed the non-specific neutralizing capacity of normal serum from man, monkey, rabbit, and guinea pig for Newcastle disease virus. Normal chicken, hamster, ferret, and mouse serum was not considered to be highly active in this respect.

Cover, et al. (1960) discussed the thermostability of chicken serum to be used in the pleuropneumonialike organism (PPLO) agglutination test. Heating the serum at 37 C for as long as 120 minutes did not influence the PPLO agglutinating power of the serum, but heating it for 10 minutes at 56 C completely destroyed the activity of the sample except in one case. The hemagglutination-inhibition titer for Newcastle disease virus was not influenced at 56 C for 20 minutes. It was emphasized that not all antibodies are stable for 30 minutes at 56 C. Previous treatment of serum by freezing or refrigeration had no influence on the effect of heating the sample. However, freezing of serum to be used in the PPLO tube or plate agglutination test increased the ability of a positive serum to agglutinate the antigen, but negative serum was unaffected by freezing.

The development of antibodies as a response to infection is closely related to the development of immunity (Hirst, 1959), but an immunological agent does not always provide protection against a specific infection because of intrinsic and extrinsic factors.

With tissue culture methods and the plaque technique for assay of viruses, it has been possible to eliminate some of the previous difficulties with studies on neutralization and to concentrate on the kinetics of antigen-antibody reactions (Hirst, 1959).

The theory of neutralization proposed by Dulbecco, et al. (1956) was made from studies of Western equine encephalomyelitis virus and poliomyelitis virus by plaque techniques and is based on the following findings:

(1) Neutralization is a direct consequence of the combination of the virus particle with antibody molecules. Indirect mechanisms, such as agglutination of the virus particles, do not play any important role, as shown by the independence of neutralization of the concentration of the virus.

(2) The kinetics of neutralization under conditions of antibody excess is of first order.

(3) The rate of neutralization, i.e., the probability for a virus particle to be neutralized per unit of time, is linearly dependent on the concentration of the antibody. Results show that the attachment of a single molecule of neutralizing antibody is sufficient to inactivate a virus particle.

(4) Under the conditions used, the virus-antibody complexes formed were very stable.

(5) A virus particle is able to combine with more than one molecule of neutralizing antibody.

(6) The characteristics of the neutralization process are independent of the cell system used for assaying the surviving virus.

On the basis of these findings, a simple model of neutralization was developed involving the following assumptions:

(1) Each virus particle possesses on its surface a number n of antigenic sites, each of which is able to undergo combination with one neutralizing antibody molecule. The stability of this combination may vary with different sera.

(2) The combination of any one site with a neutralizing antibody molecule leads to inactivation of the virus particle. The antigenic sites fulfilling these assumptions would be called critical sites.

Despite the fact that these claims made from studies of poliomyelitis virus and Western equine encephalomyelitis virus have been reiterated by Rubin and Franklin (1957) from studies of Newcastle disease virus that the antigen-antibody complex is irreversible and that a persistent fraction exists which is not neutralized by antibody, the older theory of Burnet, et al. (1937) of the reversible virus-antibody reaction cannot readily be disregarded according to Ackerman (1958).

Fazekas de St. Groth, et al. (1958a,b) pointed out errors in biometrical theory and interpretation which are believed to have confounded the results from which the theory of non-dissociation was formulated by Dulbecco, et al. (1956).

There is strong evidence that the cell-virus-antibody complex can be dissociated to reveal an active center of infection. Dissociation of the complex was examined by Mandel (1958) and found to be sensitive to a variety of influences such as salt, antibody concentration, and pH. Neutralization from its definition must be measured in terms of the capacity of the virus to infect some cell system. It is suggested

that the basic mechanism of neutralization is to prevent viral penetration of the host cell (Rubin, 1957b; Mandel, 1960).

McBride's (1959) studies on the kinetics of neutralization for antigenic analysis of polioviruses indicated that neutralization rates vary with different serum-virus combinations. Each antiserum uniquely identifies its homologous virus by neutralizing it more rapidly than any other virus, but an antiserum cannot unequivocally identify an heterologous virus. Heterologous strains of polioviruses are neutralized more slowly than is the homologous strain.

Analysis of neutralization of viruses by specific antiserums (Rubin, 1957a) has revealed unique characteristics that might serve as criteria for evaluating the effects of serum on the apparent infectivity of viral populations. These characteristics are as follows:

(1) the largely irreversible nature of the virus-antibody union under physiological conditions,

(2) the requirements for only a single antibody molecule at a viral site to cause inactivation as revealed in exponential inactivation kinetics and a linear dependence of the inactivation rate on antibody concentrations,

(3) the failure of antiviral antibody to exert any effect upon the virus once it has penetrated the cell,

(4) the lack of requirement for complement, and

(5) the absorption of antibody only with specific viral material (Dulbecco, et al., 1956; Rubin and Franklin, 1957; Rubin, 1957b).

Burnet (1959) indicated that the following statements do not seem to be invalidated by any recorded work:

(1) Virus neutralization by immune serum results from union of antibody molecules with the virus surface.

(2) These unions are of varying degrees of firmness depending on variations in population of antibody molecules, disposition and accessibility of binding sites on the viral surface, and on steric factors operating at the time of effective collision.

(3) Different strains of virus show various degrees of immunological relationship. The nature of these differences in antigen and antibody molecules is unknown and introduces further opportunity for variability when reactions are studied with antiserum not strictly homologous with the virus.

(4) With high concentration of reagents, firm union takes place and antibody absorption is demonstrable.

(5) Destruction of infectivity by adsorbed antibody will vary according to the susceptibility of the indicator host.

A more recent theory (Amelunxen and Werder, 1960) in quantitative considerations of neutralization using influenza virus, type A, strain PR8, indicates that with excess antibody in the range of complete neutralization, an average maximum of 6000 antibody molecules are bound per virus particle. In the equivalence zone, the average minimal number was 1200 antibody molecules per virus particle.

The work was predicated on nitrogen loss after serum adsorption and ultracentrifugation cycles. The nitrogen loss was assumed to be antibody molecules contained in the gamma globulin and was converted to the total number of antibody molecules bound to the total number of virus particles in the test sample. The average number of virus particles per embryo infective dose (ID_{50}) ranged from 29 to 71 as determined by ID_{50} titrations and count of virus particles by electron microscopy.

It was postulated that the probability for the inactivation of one or more critical sites on the surface of an influenza virus particle necessarily required a minimum of 1200 antibody molecules. The statistical probability for one antibody molecule to inactivate an influenza virus particle would be low.

The Neutralization Test

A. Purpose and requirements of the test.

The neutralization test is employed to measure the viral infectivity neutralizing property of antibody. It is not fundamentally different from other serological tests as all are based on antigen-antibody combinations measurable by a definite reaction or response. The test has one of four objectives (Horsfall, 1957):

(1) identification of a virus or an antibody,

(2) measurement of the concentration of antibody,

(3) determination of antigenic relatedness, or

(4) measurement of some parameter of the neutralization reaction.

The test involves titration of viral activity and the reduction of this activity as the result of combination of virus and neutralizing antibody. It is not technically difficult to perform but it does require (Horsfall, 1957; Cunningham, 1960a):

(1) standardized virus and antiserum,

(2) proper collection and handling of virus and serum to be tested,

(3) consideration of the host and its environment,

(4) a standardized quantity of inoculum,

(5) use of the optimum route of inoculation, and

(6) criteria upon which interpretation of the response is based.

Titration of virus is a quantitative assay by serial dilution, differing by a constant dilution factor, of the relationship between the amount of virus-containing material and the frequency of response of the indicator host system. The response must be characteristic of infection of the host with specific virus and one that is readily interpretable without bias (Bryan, 1957; Cunningham, 1960a).

For measurement of concentration of antibody, the practical difficulties introduced by the effects of slope of the neutralization line, host susceptibility, route of inoculation, and amount of inoculum have been reviewed by Horsfall (1957):

(1) neutralizing antibodies may be found with one set of conditions but not with another,

(2) a high neutralizing titer may emerge under some conditions and a low titer under others,

(3) with two sera from the same individual, a large increase in the amount of antibody may be indicated under some conditions and a small increase under other conditions, and

(4) comparison of antibody levels measured under different experimental conditions is not feasible without extensive and precise information about the effects of all the variables used.

Procedures involved in determining the identity, purity and potency of viral cultures and antibody require standardized quantitative methods to be reliably reproducible (Horsfall, 1957; Cunningham, 1960a). The value of any standard method is dependent upon its ability to yield data from which conclusions can be drawn that are statistically accurate.

Many of the uncertainties in interpretation can be eliminated by careful control, standardization, and quantitation

of the neutralization test. A satisfactory test should be sensitive, precise, reproducible, and, if possible, it should have a percentile end-point.

The quantitative and qualitative studies of animal virus neutralization are influenced by the heterogeneity of both antibody and virus populations (Francis, 1959; Burnet, 1959).

Despite the extensive use of neutralization tests in diagnostic laboratories, very little has been effectively established about the nature of the process by which infectivity of animal viruses is destroyed (Burnet, 1959).

B. Some variables in the neutralization test for IBV.

No single method has yet been devised and universally accepted for the study of viral neutralization by antibody (Lennette, 1956; Cunningham, 1960a,b). Among the variables encountered in the methods employed by different laboratories using the neutralization test for IBV are variations in:

(1) strain of the antigen used,

(2) titer of the antigen used,

(3) use of virus-dilution or serum-dilution techniques,

(4) time of collection of antiserum for desired serological analysis,

(5) heat-inactivation or non-inactivation of antiserum prior to storage and/or use,

(6) time and temperature of storage of antiserum,

(7) storage of antigen or antiserum in liquid or desiccated state,

(8) kind and pH of diluent used for preparing virus or serum dilutions,

(9) kind and concentration of antibiotics used in the diluent,

(10) ratio of volumes of serum and antigen used in the test,

(11) time and temperature of incubation of serum-virus mixtures,

(12) use of different cultural mediums such as animals, embryonating chicken eggs, or cell and tissue cultures.

(13) amount of inoculum and route of inoculation,

(14) criteria used to determine a positive response of the cultural medium to infection by the virus,

(15) method used to calculate the end-points of viral activity, and

(16) method used to calculate the neutralization index.

Variation in antigenic types.

Recognition of serological variation among the animal viruses is now the rule rather than the exception as was true twenty-five years ago (Francis, 1959).

Two strains of IBV widely used in research laboratories are the Massachusetts strain which is virulent to chicks and may be

used in chicken challenge tests for evaluation of efficacy of IB vaccine, and the Beaudette strain which has been completely embryo-adapted by numerous serial passages through embryos so that it is lethal to chick embryos but non-lethal, and non-immunogenic to chickens. The Beaudette strain is employed by many laboratories as the antigen in the neutralization test for assay of antibodies produced by IBV. Other strains of IBV that are attenuated, and thus less virulent for chickens but still capable of producing an immune response, are used in vaccine production. These include the Connaught (Crawley) strain, DG (New Hampshire), Wachtel, and other strains.

Another strain of IBV often used as an antigen in the test for neutralizing antibodies is the DA (New Hampshire) strain which has also undergone many serial passages through embryos and has several characteristics similar to those of the Beaudette antigen.

Jungherr, et al. (1956a,b) reported a strain of IBV which appeared to be a different immunogenic type than the strains previously reported. This strain is now referred to as the Connecticut (A5968) strain.

Hofstad (1958; 1959b) reported that certain isolates of IBV when compared by reciprocal serum neutralization tests have antigenic differences. These three strains (isolates 97 and 609 by Hofstad, and A5968 by Jungherr) appear to be entirely

different from the standard strains and from each other based on results of the neutralization tests.

The Massachusetts strain induces antibodies that are readily detected by the Beaudette strain, but the Connecticut strain and the IBV isolates identified as 97 and 609 by Hofstad (1959b) induce antibodies that are not readily detected by heterologous strains of IBV or by the embryo-adapted Beaudette strain.

Raggi and Raymond (1960) attempted differentiation of six IBV strains by using the pathogenicity of the virus for chicken embryos as the criterion. Lack of a uniform death pattern and inconsistent pathogenicity for embryos prevented such a differentiation from being used as a method for identifying various virus strains.

Hofstad indicated that the original Beaudette strain appears to be a suitable virus to use in the test for diagnosing past infection by IBV although the NI does not equal that obtained with an homologous virus and its antiserum. The work reported by Jungherr, et al. (1956a,b) indicates that the Beaudette strain should not be used against a known Connecticut antiserum in the test for neutralizing antibodies.

Considerable variation may be detected in the NI of antigenically related isolates (Cunningham, 1960b; Raggi and Lee, 1957). The plurality of these strains indicates consideration

of their properties for evaluation of immunity induced by IB vaccines.

Variation in titer of the antigen used in the test.

The Beaudette antigen is widely used and gives consistent results when used with antigenically related strains of IBV. However, it does require careful handling. Hofstad pointed out that it is difficult to maintain a high titer when handled in the same manner as other isolates of IBV. This strain should have a titer of at least 10^7 ID_{50} per ml (Manual for the Examination of Poultry Biologics, 1959) and be lethal to nearly all infected chick embryos by the third day after inoculation. If the virus has a lower titer, the range of the NI becomes limited so that the test results are inconclusive.

Titration of IBV in the neutralization test indicates only the number of infectious units and not the total actual particles of which some may be infectious and others non-infectious. The size of the inoculum used can affect the amount of non-infectious virus available to combine with antibody, but is not reflected in titrations of infectivity (Francis, 1959).

The infective property of a virus is its most subtle and unstable character (Horsfall, 1957). At 35 C the half-life of an infective particle is in many instances surprisingly brief. With influenza and mumps viruses it can be less than 2 hours, and with so-called stable viruses, such as Newcastle, poliomyelitis, and vaccinia, it is rarely longer than 24 hours.

Simpson and Groupé (1959) indicated that the temperature of incubation may be a critical factor in the behavior of IBV in chicken embryos. The Beaudette strain was lethal for embryos incubated at 34 C or 38 C and was pathogenic for suckling mice following intracerebral inoculation. After twenty-five serial passages in suckling mice, the original population had been replaced by one that was lethal for embryos incubated at 34 C but not at 38 C, the apparent optimum temperature for this virus.

Page (1954) and Page and Cunningham (1960) reported that the neutralization test for IBV should be performed at 4 C when incubating serum-virus mixtures before inoculating 9- to 11-day embryonating eggs, and that the virus-control mixture should be inoculated last.

Hofstad (1956, 1958) indicated that the Beaudette strain of IBV is difficult to maintain at high titer and that thermal inactivation or infectivity is rapid.

Page and Cunningham (1960) reported the rate of inactivation of the Beaudette strain to be $10^{0.26}$ per week at 4 C, $10^{0.31}$ per day at 25 C, and $10^{0.14}$ per hour at 37 C.

Ozawa (1959) indicated that the viral infectivity of IBV propagated in isolated chorioallantoic membrane decreased at the rate of $10^{0.63}$ per hour at 37 C and at $10^{0.113}$ per week at -25 C.

Singh (1960) reported that the Beaudette strain consists primarily of thermolabile D phase virus particles. The infectivity of this strain decreased $10^{1.3}$ in 3 hours at 37 C.

Use of virus-dilution or serum-dilution techniques in the test.

With most viruses, accurate quantitation of neutralizing antibody can be made in tissue culture systems by the constant virus-varying serum (serum-dilution) method (Lennette, 1959). With some viruses, notably IBV, it is difficult to establish a constant number of doses due to the relative instability of the virus and other intrinsic characteristics. These characteristics of IBV make the use of the varying virus-constant serum (virus-dilution) method the method of choice when chicken embryos are used and gives a useful numerical value as an index of neutralization. One report (Crawley, 1951) indicates that the serum-dilution method may be used for survey purposes.

Both methods give sensitive and reproducible results but the numerical values obtained by one method are not directly correlated with those obtained by the opposite method.

Variation in time of collection of antiserum for desired serological analysis.

The neutralizing antibody response following infectious bronchitis infection shows that antibodies at low level can be detected as early as ten to fourteen days after a single inoculation of young chicks with IBV.

The antibody level rises rapidly during the next four weeks, reaching a plateau at six to eight weeks than gradually declines (Hipolito, 1955). Dimopoullos and Cunningham (1956) reported that a second inoculation of young adult chickens about twelve weeks after the initial inoculation stimulated an additional rise in the antibody level above that of the first plateau. Antibodies remained at a high level for as long as twenty weeks after the initial inoculation.

Investigations by Page (1950) and Fabricant (1951) indicated that quantitative assay of serum for antibodies against IBV at an NI of 2.0 or higher should not be attempted before fourteen days after exposure to IBV.

Variation in criteria used to determine a positive response in the chicken embryo to infection by various strains of IBV.

The criterion of viral activity of a positive response of the host as a manifestation of successful infection is indicated by death or signs of infection characteristic of the virus. These signs in the surviving embryos may include stunting, dwarfing, curling, clubbed down, thickened amnion, and foci of urate-like deposits in the kidneys.

With the Beaudette strain of IBV, the criterion for determining the positive response in chicken embryos can be based solely on embryo lethality by the fourth day after inoculation. Rarely are other signs of infection noted in the

surviving embryos. With other strains of IBV less well adapted to embryo culture, use of other signs of infection is necessary to obtain a true virus titer. Evaluation of these additional criteria may vary widely among different laboratories.

The criteria often used as evidence of infection of chicken embryos by strains of IBV that have undergone a few embryo passages are as follows:

(1) death of embryo between the third and seventh day after inoculation;

(2) stunting and dwarfing of the embryo so that a tightly curled embryo is produced;

(3) the amnion is thickened and restricts the movements of the embryo;

(4) immature feathers (clubbed down); and

(5) foci of urates in the kidney.

By the seventh day after inoculation with IBV, the embryo is stunted to about one-half normal size (Loomis, et al., 1950). Some strains in high dilutions may produce a loosely curled embryo with slight stunting, that is, its weight may be slightly more than 25% below that of a normal embryo incubated at the same time.

For example, chicken eggs weighing 24 ounces per dozen normally have embryos weighing about 18 grams on the 17th day of incubation. By the seventh day after inoculation of

ten-day old embryonating eggs, a 25% difference in weight (or less than 15 grams) from that of normal embryos incubated at the same time is used as evidence of infection and may be considered as a positive response of the embryo.

If the stunting of embryos were caused only by IBV, then the use of the 25% weight differential would simplify selection of this criterion for indicating positive responses of the embryos to infection by various strains of IBV. Stunting of non-infected embryos may be caused by improper temperature and humidity prior to and during incubation. Bacterial infection of the embryo may also cause stunting. Irregular turning of eggs during the pre-inoculation period of incubation can have an adverse effect on the embryo.

According to Hitchner and White (1955), the presence of focal depositions of urate-like material in the kidney is found with the Connaught strain. This finding is not always present but when found is considered pathognomonic for IBV infection of the chick embryo.

Obviously, such a wide range in the selection of signs of infection used to indicate a positive response of the embryo to infection with IBV allows considerable latitude in the choice of criteria for determining those which are diagnostic for the strain of IBV used in the neutralization test performed by various laboratories.

In Methods for the Examination of Poultry Biologics (1959), it is demonstrated that the range of infective doses (ID_{50}) from virus titration may vary from $10^{2.6}$ to $10^{8.3}$ per ml according to the choice of criteria used to select positive embryo responses to infectivity.

For example, at seven days after inoculation, the following may be used in calculating the virus titer:

Death of embryo = $10^{2.6}$/ml

plus

Curling and stunting = $10^{6.0}$/ml

plus

Clubbing of down = $10^{7.0}$/ml

plus

Urates in kidney = $10^{8.3}$/ml

The gross lesions produced by low embryo passages of Connecticut and Massachusetts strains of IBV generally confirm the range demonstrated in this sample according to unreported data accumulated by the author.

Since a single lesion found in most disease conditions is rarely considered diagnostic for a specific entity, it appears that the finding of at least two signs of infection in the surviving embryos would make the ID_{50} titers reported by different laboratories more uniform.

Variation in methods used to determine the end-point of viral activity.

Identity of viral activity is referred to as the unit which produces the positive response. There are numerous designations characteristic of the reaction, the most common being lethal dose (L.D.) and infectious dose (I.D.). With the embryo-adapted Beaudette strain, the L.D. and I.D. are the same, i.e., infectivity is manifested as lethality. With strains less well adapted to embryo culture, the L.D. and I.D. do not follow this convenient pattern as lethality is not a constant or uniform finding and cannot be used as the sole criterion of infection. In such cases the criteria of gross pathological alteration must be utilized as well as mortality of the embryo. For uniformity of expression, I.D. should be used for all strains of IBV.

In some instances, the end-point of viral infectivity is considered to be the highest dilution of the virus in which 50% or more of the embryos show positive responses. It is evident that an end-point derived on this basis is only an approximation. The more desirable evaluation is the 50 per cent end-point method of Reed and Muench (1938) whereby the end-point is determined by interpolation from cumulative frequencies and is expressed as the ID_{50}. In this fashion, the end-point of viral activity can be calculated more accurately for the virus-serum and virus-control mixtures.

Variation in calculation of the neutralization index.

The neutralization index (NI), a measure of the reduction of viral activity by neutralizing antibody, is the difference between the virus titer and the serum titer with both titers calculated by the same method.

Expressed in another manner, it is the reciprocal of the difference between the end-point of viral infectivity of the virus-control mixture and that of the serum-virus mixture.

The virus-control mixture should be inoculated after all serum-virus mixtures have been inoculated to take into account any deleterious effects of time and temperature on the virus antigen.

Some laboratories use a normal, or pre-infection, serum in lieu of a diluent in the virus-control mixture. In this case the NI is the reciprocal of the difference between the test serum and that of the normal serum titer.

The NI can be expressed as $\log_{10}$ ID_{50} NI or as its anti-logarithm to express the neutralizing doses in arithmetic terms (Cunningham, 1960a; Lennette, 1956; Rhodes and van Rooyan, 1953; and Methods for the Examination of Poultry Biologics, 1959).

The $\log_{10}$ ID_{50} NI of normal chicken serum is not expected to exceed 1.5, or 36 neutralizing doses (Cunningham, 1951). According to Methods for the Examination of Poultry Biologics

(1959), serum collected from vaccinated chickens should contain at least 1000 (10^3) neutralizing doses in 80% of the samples, or a $\log_{10}$ ID_{50} NI of at least 3.0 in 80% of the samples.

MATERIALS AND METHODS

Vaccines

Several serial lots of infectious bronchitis vaccines from fourteen licensed manufacturers were used. These vaccines included both the single component vaccine, Infectious Bronchitis Vaccine, live virus, and the combined vaccines, Newcastle Disease Vaccine, B_1 type and Infectious Bronchitis Vaccine, live virus, and Newcastle Disease Vaccine, B_1 type, and Infectious Bronchitis Vaccine, and Fowl Laryngotracheitis Vaccine, live virus.

Viruses for production of specific antiserums.

1. The Massachusetts strain isolated and maintained in continuous chicken passage by Dr. H. Van Roekel, University of Massachusetts, was used for immunizing chickens for production of specific antiserum. This strain is identified as IB-41 at the IBV Repository, Michigan State University, East Lansing, Michigan. The sample received from the Repository had been through four chicken embryo passages, one chicken passage, and two additional embryo passages. After receipt from the Repository, the strain was then passaged once in embryos and three times in 4- to 6-week old chickens.

The lower trachea and lungs harvested from the inoculated chickens were minced in a TenBroeck tissue grinder, pooled

with an equal quantity of nutrient broth (Difco)* containing penicillin (Squibb)** and dihydrostreptomycin (Squibb), 1000 units and 2 mg per ml, respectively, and stored at -60 C in 3 ml amounts in two-dram screw-cap vials. At the time of use the material was thawed rapidly in cool tap water, centrifuged at 4 C for 5 minutes at 2000 rpm in a Model PR-1 centrifuge# to sediment the coarse particles, and the supernatant fluid was used as inoculum for chickens.

2. One of the commercial vaccines which was reported to contain the Connecticut strain identified as A5968 by Jungherr, et al. (1956b), was used for immunizing chickens for production of specific antiserum.

Viruses for use as antigens in the neutralization tests.

1. The Beaudette embryo-adapted strain identified as IB-42 at the Repository was supplied by Dr. C. H. Cunningham. It had been maintained by repeated serial passage every 30 to 60 days in ten-day old embryonating chicken eggs which had received 0.2 ml of a 10^{-2} dilution of the previous passage of the virus. The infected allantoic fluids from living embryos were harvested at 25 to 30 hours post-inoculation when

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* Difco Laboratories, Inc., Detroit, Michigan.

** E. R. Squibb and Sons, New York, N. Y.

\# International Equipment Co., Boston, Mass.

about one-third of the embryos appeared near death. The eggs were chilled at 4 C for two to four hours. The fluids were then harvested with a 10 ml glass syringe, pooled in an Erlenmeyer flask kept in an ice bath, and mixed thoroughly before dispensing 3 ml amounts into one-dram screw-cap vials for storage at -60 C. Sterility tests of fluids from individual eggs and from the pooled batch were made using NIH thioglycollate broth medium (Difco).

At the time of use in a neutralization test, the virus was quickly thawed in cool tap water and centrifuged at 2000 rpm for 10 minutes at 4 C to sediment the small amount of insoluble precipitate formed upon thawing. The supernatant fluid was used as the antigen for the neutralization test and the remaining portion of the material was discarded.

2. The Connecticut strain was isolated from the same commercial IB vaccine used for immunizing chickens for the production of specific antiserum. This virus was passed through chicken embryos an additional seven to eleven times in a manner similar to that described for the Beaudette strain.

Eggs used for inoculation of viruses.

Eggs from Newcastle disease and infectious bronchitis susceptible White Leghorn hens from one commercial hatchery were used. Incubation of the eggs prior to and after inoculation with IBV was at 37.5 C (99.5 F) in an electric, forced-

draft incubator with controlled humidity and an automatic device for turning the eggs every two hours. Inoculation of nine- and ten-day old embryonating eggs with IBV was via the allantoic cavity. A single hole was drilled about one-fourth inch above the base of the air cell and about one-half inch away from the embryo so that an area devoid of blood vessels was available over the allantoic cavity. After the hole was swabbed with tincture of metaphen, a 5/8-inch, 27-gauge needle on a 1 ml B-D Yale glass tuberculin syringe was inserted full length and the inoculum deposited. After inoculation, the hole was sealed with melted paraffin.

Diluent.

Nutrient broth (Difco) was used throughout the study. With few exceptions, crystalline penicillin G potassium (Squibb) and crystalline dihydrostreptomycin sulfate (Squibb) was added to the diluent so that the final concentration of antibiotics was 1000 units and 2 mg per ml, respectively. Filtered normal horse serum* was added to the diluent in a final concentration of 2% and used in a few neutralization tests.

Vaccination of chickens.

Twelve lots of 100 White Rock one-day old chicks from non-vaccinated flocks of the same commercial hatchery were used.

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* Colorado Serum Co., Denver, Colorado.

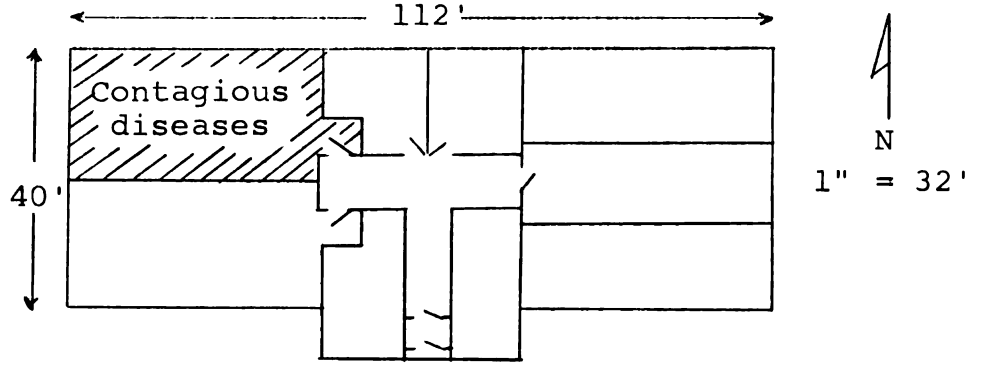
Chicks of the same lot were hatched at the same time and are referred to as hatchmates.

During the period before vaccination, the chicks were kept in isolation in an electric brooder battery in Room 1 in an isolated research building (figure 2). Air from each room was exhausted by negative pressure through a duct to the outside of the building. Two changes of coveralls and boots were required for the caretaker and observer to enter the rooms. All rooms were cleaned and disinfected with lye water between use by each lot of chicks.

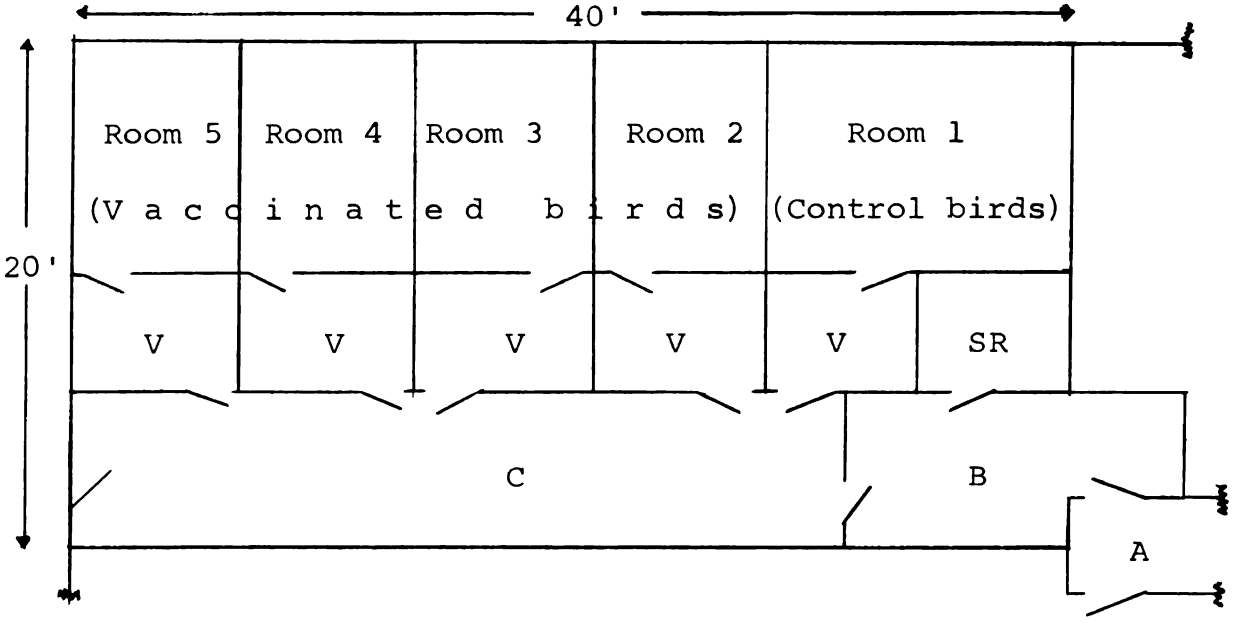
When the chicks were 15-days old, each lot was divided into five groups (A, B, C, D and E) and individually wing-banded. Groups A, B, C and D were transferred to individual isolation Rooms 2 to 5. The chicks were vaccinated by the intranasal drop method with different serials of commercially available IB vaccines. As they were vaccinated, they were placed on the floor with wood shaving litter and with free access to feed and water. An electric heat lamp was used in each room to supplement the hot-water radiator system. Each vaccinated group was observed several times a week until the first collection of serum at 21 days after vaccination.

Group E was retained in the battery in Room 1 as non-vaccinated controls for that lot of hatchmates.

An exception to the regular procedure of handling and



Plot Plan - Animal Disease Research Building No. 5.



Floor Plan - Contagious Diseases section of building No. 5.

(Scale: 1" = 8')

Legend:

A - Entrance hall.
B - Anteroom for removing street clothes.
C - Inside hall.
SR - Free-flowing steam room.
V - Vestibule to each room--contains feed, water, coveralls, boots, and disinfectant pail with boot brush.

Figure 2. Floor plan of isolated research building showing security and location of isolation rooms where chickens were vaccinated with infectious bronchitis vaccines.

vaccinating chicks at the research building occurred when a large part of the non-vaccinated chicks of group 10E was transferred to Horsfall-Bauer type stainless steel isolation units in an isolation room of another building. These isolation units were self-contained and arranged in two tiers of six units each. All air taken into each of these units was drawn from the room through a glass wool filter and exhausted by negative pressure through another filter into a duct system which led to a point at least 100 feet from the air intake into the building. Feed and water were introduced through separate one-inch tubes from the top of each unit directly into the feed or water pan. Supplementary heat, when required, was thermostatically controlled and supplied by electric heat cables on the inside walls of each unit. Light was supplied by an electric light bulb inside each unit. Each unit accommodated two or three young adult chickens or ten chicks up to four weeks of age.

The chickens of group 10E were inoculated with the Connecticut and Massachusetts strains of IBV, respectively, to produce specific antiserums and were re-identified as 10MC and 10M, respectively (Tables 3, 4 and 5). Inoculation of each chicken was by the intranasal and ocular drop method. The chickens were held in the door of each unit when inoculated so that negative air pressure carried extraneous aerosols directly

into the unit without contaminating other units in the room. Observation of the two or three chickens in each unit was made daily through the two windows of the unit.

Seven chickens also of group 10E were kept as controls and were distributed into four adjacent Horsfall-Bauer type units and bled at irregular intervals. Neutralization tests were performed to demonstrate the lack of cross-contamination by the two virus strains.

Collection of serum.

Serum from chicks of all groups was used in the evaluation program assigned by the Veterinary Biologics Licensing and Inspection Sections of the United States Department of Agriculture. Serum from only portions of these groups was used to investigate the serological variables encountered in neutralization test procedures reported in this study.

At 21 and 28 days after vaccination, ten chicks from each vaccinated group, except groups 10MC and 10M, were bled by cardiac puncture in the inside hallway of the building (figure 2). The chicks of group 7A were bled additional times according to the schedule given in Table 2. The control chicks (group E of each lot) were bled at the time of vaccination of the groups and again 21 days later to demonstrate their susceptibility to IBV. The chickens in groups 10MC and 10M were bled by cardiac puncture at the Horsfall-Bauer type isolation

units according to the schedule given in Tables 3, 4 and 5.

About 6 to 10 ml of blood was individually collected in the morning with a 2 1/2-inch, 20-gauge needle on a 10 ml glass Luer-lock syringe and allowed to clot on a long slant in a sterile test tube. Sufficient serum was usually available for testing purposes by the end of the day and was frequently processed and stored on the same day that the birds were bled.

Blood collected from the individual chicken remained at room temperature for about eight hours. The serum was then poured off the clot into individual sterile test tubes and centrifuged at 750 rpm for 15 minutes at 4 C for clarification.

Equal amounts of serum from two chickens was pooled in a two-dram screw-cap vial. Each vial contained about 3 ml of serum. In several instances, duplicate serum samples were prepared. One sample was not heated, while the other sample was subjected to 56 C for 30 minutes. Sometimes both duplicate samples were or were not heated. Either one or both vials were then stored at 4 C or at -27 C. In other instances a pooled sample was prepared from two chickens and individual samples from the same two chickens were also prepared and stored.

At the time of testing, the frozen serum was thawed and centrifuged at 1000 rpm for 5 minutes to sediment the flocculate formed on thawing. A few previously non-heated samples were subjected to 56 C for 30 minutes after thawing for comparative studies with its duplicate sample.

Procedure for production of specific antiserum.

The study of antigens as a variable in the neutralization test was divided into two parts, each of which was conducted with two groups of chickens:

1. Connecticut antiserum tested with Beaudette antigen and the homologous Connecticut antigen.
   a. Twenty-four chicks 15-days old (group 7A) were vaccinated with a serial of a commercial IB vaccine known to contain the Connecticut strain of IBV. The chicks were re-vaccinated with the same serial 28 days later. At intervals of 21, 28, 42, 49 and 56 days after the original vaccination, about 9 ml of blood was individually collected from ten of the vaccinated chickens. Serum from only two chicks was used in this study (Table 2). Equal amounts of non-heated serum from the two chicks were pooled and stored at -27 C.
   b. Three chickens 11-weeks old (group 10MC) were vaccinated and then re-vaccinated 13 and 36 days later by the intranasal and ocular routes using the same serial of commercial IB vaccine. At the intervals indicated in Table 3, 20 to 40 ml of blood was collected from each chicken. The serum

from all vaccinated chickens at each bleeding was pooled and a representative non-heated sample was stored at -27 C until tested with both antigens.

2. Massachusetts antiserum tested with the Beaudette antigen and the heterologous Connecticut antigen.
   a. Four chickens 4 1/2-weeks old (group 10M-#1) were inoculated several times and bled according to the schedule in Table 4.
   b. Four chickens 11-weeks old (group 10M-#2) were inoculated several times and bled according to the schedule in Table 5. Infected fluids containing the Massachusetts strain of IBV were administered by the intranasal and ocular routes to both groups 10M-#1 and 10M-#2. Serum from these groups was prepared and tested in the same manner as for 1b above.

At the end of the bleeding schedules for groups 10MC, 10M-#1, and 10M-#2 (Tables 3, 4 and 5), all serum collected was pooled, Seitz-filtered, and dispensed into screw-cap vials of 5 ml each for storage at -27 C. A representative non-heated sample was stored at -27 C until tested with both antigens.

Procedure for comparison of neutralization indices after various methods of handling serum samples.

Serum samples prepared in duplicate as described in Collection of serum were used in this portion of the study of serological variables in the neutralization test.

1. Neutralization indices of individual serum and pooled serum of the same two chickens (Table 9, figure 9).
2. Neutralization indices of serum collected from the same chicken(s) 21 and 28 days after a single intranasal administration of different serials of IB vaccines to 15-day old chicks (Table 10, figure 10).
3. Neutralization indices after various methods of processing and storing serum samples. This section was divided into three parts:
   a. Neutralization indices from duplicate serum samples where one serum was subjected to 56 C for 30 minutes, the other non-heated, and both samples stored at 4 C (Table 11, figure 11).
   b. Neutralization indices from duplicate serum samples where one serum was subjected to 56 C for 30 minutes, the other non-heated, and both samples stored at -27 C (Table 12, figure 12).
   c. Neutralization indices from duplicate serum samples where one serum was stored at 4 C and the other serum at -27 C (Table 13, figure 13).

Collection of trachea.

This experiment, similar to the procedure described by Jungherr, et al.(1956b), was designed to evaluate the response of the tracheal mucosa following a single intranasal vaccination of eight groups of 15-day old chicks with serials of commercial IB vaccines from eight different manufacturers. Five groups each received a single component IB vaccine and three groups each received a combined Newcastle-Bronchitis vaccine. The infectivity titer of each single component IB vaccine was determined, but the infectivity titer of the combined Newcastle-Bronchitis vaccines was not determined. It was impractical to selectively separate the IBV fraction of the combined vaccine to obtain a true quantitative titer for IBV. An optional method would be to determine the titer of a vial of single component IB vaccine produced from the same batch and desiccated at the same time as the IB vaccine used for the combined Newcastle-Bronchitis vaccine. This was not available for testing.

The vaccinated groups of about 22 chicks each were designated 6A, 6B, 6C, 6D, 7A, 7B, 7C and 7D. Groups 6E and 7E were retained as controls for their respective lots of hatchmates.

Transverse sections of the mid-portion of the trachea from two chicks in each vaccinated group were collected every third

day through the twenty-first day and also on the twenty-eighth day after vaccination. Eight to ten chicks of each group were bled 21 and 28 days after vaccination to obtain a NI for each group for correlation with the histopathological changes observed following vaccination.

Sections of trachea were also collected from two chicks of groups 6E and 7E when they were 15 and 36 days old. This corresponded to the day of vaccination (0 day) and day of bleeding (21st day) for the vaccinated groups.

Specimens were placed in Zenker's solution at the time of collection. About twenty hours later they were placed in running tap water for 24 hours, then stored in 80 per cent alcohol until the tissue could be embedded in paraffin, cut and stained with hematoxylin and eosin.

After microscopic examination of the sections, an arbitrary evaluation of the response of the tracheal mucosa was made (figure 14) ranging from 0 (normal) through I, II, III and IV (the maximum response).

Neutralization test procedure.

The virus-dilution method using nine- and ten-day old embryonating chicken eggs as the indicator system was employed.

Before the virus antigen was thawed for use in the test, all tubes were labelled, 0.5 ml amounts of serum and diluent pipetted into the appropriate tubes (flaming of the mouth of

the test tube as part of the aseptic procedure occurred at this stage but not after the virus antigen was placed in the test tube), embryonating eggs labelled and prepared, and the test tube rack placed in an ice bath.

Serial ten-fold dilutions of IBV-infected allantoic fluids were prepared in nutrient broth containing antibiotics and mixed with equal volumes of undiluted serum as described by Cunningham (1960a) with the following modifications in technique:

1. The virus antigen was prepared for use as previously described under the Beaudette antigen in Viruses for antigens in the neutralization test.
2. A 1.0 ml serological pipette was used to place 0.5 ml of the virus in 4.5 ml of diluent in the first dilution tube.
3. A separate 2.0 ml serological pipette was used to mix the virus by aspiration and expelling the mixture 15 times. After 0.5 ml of the mixture was transferred to the next dilution tube, the pipette was discarded. Another 2.0 ml pipette was used to mix and transfer the next dilution, etc.
4. Starting with the highest dilution of virus prepared, a 2.0 ml serological pipette was used to transfer 0.5 ml of that dilution to each tube containing the respective serum and diluent for that dilution. A separate

2.0 ml serological pipette was used for each virus dilution.

This is an exception to the procedure where an individual pipette is used to transfer the highest to lowest virus dilutions to all tubes for one serum before proceeding to all tubes for the next serum.

5. The tubes containing the serum-virus and virus-control mixtures were agitated several times by hand at the time the virus was added. After virus had been added to all tubes the tube rack was then shaken.
6. With few exceptions, the mixtures containing virus were kept in an ice bath until the eggs were inoculated.

Each of the five eggs used per dilution received 0.1 ml of inoculum beginning immediately after shaking the test tube rack unless an incubation period of the serum-virus mixtures was specified for an occasional test. A separate syringe was used to inject each serum-virus and virus-control series beginning at the highest dilution of that mixture.

Embryo mortality found by candling at 18 to 24 hours post-inoculation was considered to be due to non-specific causes and was not included in calculating the final results. Embryo deaths after the first day were recorded daily and were considered to be due to viral activity. All survivors were

examined on the seventh day for gross signs of infection characteristic of IBV (Loomis, et al., 1950; Hitchner and White, 1955).

The infective dose (ID_{50}) titer for each serum-virus and virus-control mixture was calculated according to the method of Reed and Muench (1938) to the nearest centile. The neutralization index (ID_{50} NI) for each serum was the reciprocal of the difference between the end-point of the viral infectivity of the serum-virus mixture and the end-point of the virus-control mixture. For brevity the $\log_{10}$ ID_{50} NI will be referred to hereafter in the text as the NI.

For most neutralization tests, the titer of the virus-control mixture for both the Beaudette and the Connecticut antigens ranged from $10^{6.0}$ to $10^{8.0}$ per 0.1 ml of inoculum. If the titer were below $10^{6.0}$ per 0.1 ml, the range of the NI appeared to be restricted and was considered of doubtful value.

In addition to accuracy with the pipette and syringe, speed in handling the virus was stressed (Table 6). Most tests had from 3 to 7 series of mixture tubes and an average of about 75 minutes was required from the moment of thawing the virus antigen until the inoculated eggs were returned to the incubator.

Normally the virus-control mixtures were inoculated into eggs last to take into account any deleterious effect of time and temperature on viral infectivity.

A series of tests was conducted by titrating the virus-control mixtures at the beginning and at the end of each neutralization test. Two types of diluents were used: (1) nutrient broth containing antibiotics, and (2) nutrient broth containing antibiotics with filtered normal horse serum in a final concentration of 2% (Tables 7 and 8). All mixtures were kept in an ice bath at 4 C except for one test which was conducted at room temperature (20 C).

RESULTS

The results are incorporated into three major parts--Antigens, Serology, and Histopathology--for evaluation of certain variables encountered in the neutralization test for assay of immunity induced by commercial infectious bronchitis vaccines.

A. Antigens

This portion is divided into (1) comparison of the Beaudette and the Connecticut antigens as variables in the neutralization test when tested against specific antiserums, and (2) the influence of time and temperature on the inactivation of the Beaudette antigen during the neutralization test.

1. Comparison of the Beaudette and the Connecticut antigens when tested against specific antiserums in the neutralization test.

With normal chicken serum, the commonly accepted NI does not exceed 1.5. Antibody against the Connecticut strain of virus was below this maximum NI as determined by the Beaudette antigen for at least 68 days after initial vaccination, although the chickens had been re-vaccinated several times by the intranasal route (Tables 2 and 3; figures 3 and 4).

When the homologous Connecticut antigen was used with antibody against the Connecticut strain of virus, the NI ranged

from at least 2.6 at the first bleeding 21 days after vaccination to as much as 8.2 at least 53 days after vaccination.

Antibody against the Massachusetts strain of virus produced in two different age groups of hatchmates had an NI, as determined by the Beaudette antigen, which ranged from 3.2 to 6.2 throughout the periods of 69 and 117 days each (Tables 4 and 5; figures 5 and 6).

When the heterologous Connecticut antigen was used with antibody against the Massachusetts strain of virus, the NI ranged from 1.7 to 4.8 but in each instance the NI was higher when the Beaudette antigen was used.

Two groups of pooled, Seitz-filtered serum containing antibodies against the Massachusetts strain of virus had neutralizing indices of 5.2 and 5.5 as determined by the Beaudette antigen and neutralization indices of 2.0 and 3.8 as determined by the Connecticut antigen (Tables 4 and 5).

Pooled, Seitz-filtered serum containing antibodies against the Connecticut strain of virus was not tested with either antigen.

Serum from control chickens (groups 7E and 10E) had an NI below the commonly accepted NI of 1.5 for normal serum at all times (Tables 2, 3, 4 and 5).

Besides the effect of strain differences of the antigens when used against specific antiserums, the titer of the virus-

control mixture used in the test may influence the NI obtained for each serum. As indicated in Tables 2, 3, 4 and 5 and figures 3, 4, 5 and 6, the use of a virus with a low titer often resulted in a low NI for the serums in that test. With a virus of a higher titer, a higher NI was usually obtained.

From these tables it would appear that a virus-control titer below $10^{6.0}$ per 0.1 ml would limit the NI to a value below that which might be possible.

2. Influence of time and temperature on inactivation of the Beaudette antigen during the neutralization test.

The average technician uses much more time than is usually realized if time intervals are recorded from the instant a vial of frozen virus is removed from storage until the inoculated eggs are returned to the incubator. The results of eight tests and the average time required for each step are presented in Table 6. Tests 1 and 2 were planned to have the serum-virus mixtures incubated for 30 minutes at 4 C before the eggs were inoculated. With the other tests, no definite time of incubation was planned and inoculation of eggs was inaugurated as soon as all virus dilutions had been transferred to their respective serum tubes for mixing.

The time that the serum-virus mixtures were incubated at 4 C before inoculation of eggs ranged from 6 to 48 minutes with an average of 25 minutes even though no incubation period was specified in 6 of the 8 tests.

TABLE 2

Neutralization tests using the Beaudette and the Connecticut antigens with serum produced in chicks (group 7A) vaccinated intranasally at 15 days of age with a commercial vaccine known to contain the Connecticut strain of IBV.

Pooled serum from bird number	Days after initial vaccination	Neutralization test results	
		Beaudette antigen (V - S = NI) *	Connecticut antigen (V - S = NI) *
Control group 7E (5 chicks)			
(2 untagged birds)	(0)	6.83 - 6.38 = 0.45	. . .
341 & 342	(21)	6.83 - 6.50 = 0.13	. . .
343 & 344	(21)	. . .	5.8 - 5.63 = 0.17
Vaccinated group 7A (24 chicks)			
520 & 530	21	6.63 - 5.80 = 0.83	4.63 - $\leq$2.0 = $\geq$2.6
520 & 530	28**	6.63 - 5.50 = 1.13	7.00 - $\leq$0.63 = $\geq$6.3
520 & 530	42	6.63 - 5.17 = 1.46	5.8 - 1.17 = 4.63
520 & 530	49	6.63 - 6.17 = 0.46	7.00 - 2.17 = 4.83
#532 & 536	56	5.50 - 4.84 = 0.66	5.8 - 1.32 = 4.48

* In this and all succeeding tables, virus-control titer (V) minus serum-virus titer (S) equals $\log_{10}$ ID_{50} neutraliztion index (NI).

** Group was re-vaccinated on 28th day with same serial of vaccine.

\# Equal portions of serum from these birds was used on 56th day as serum from previous birds was no longer available.

. . . Not tested.

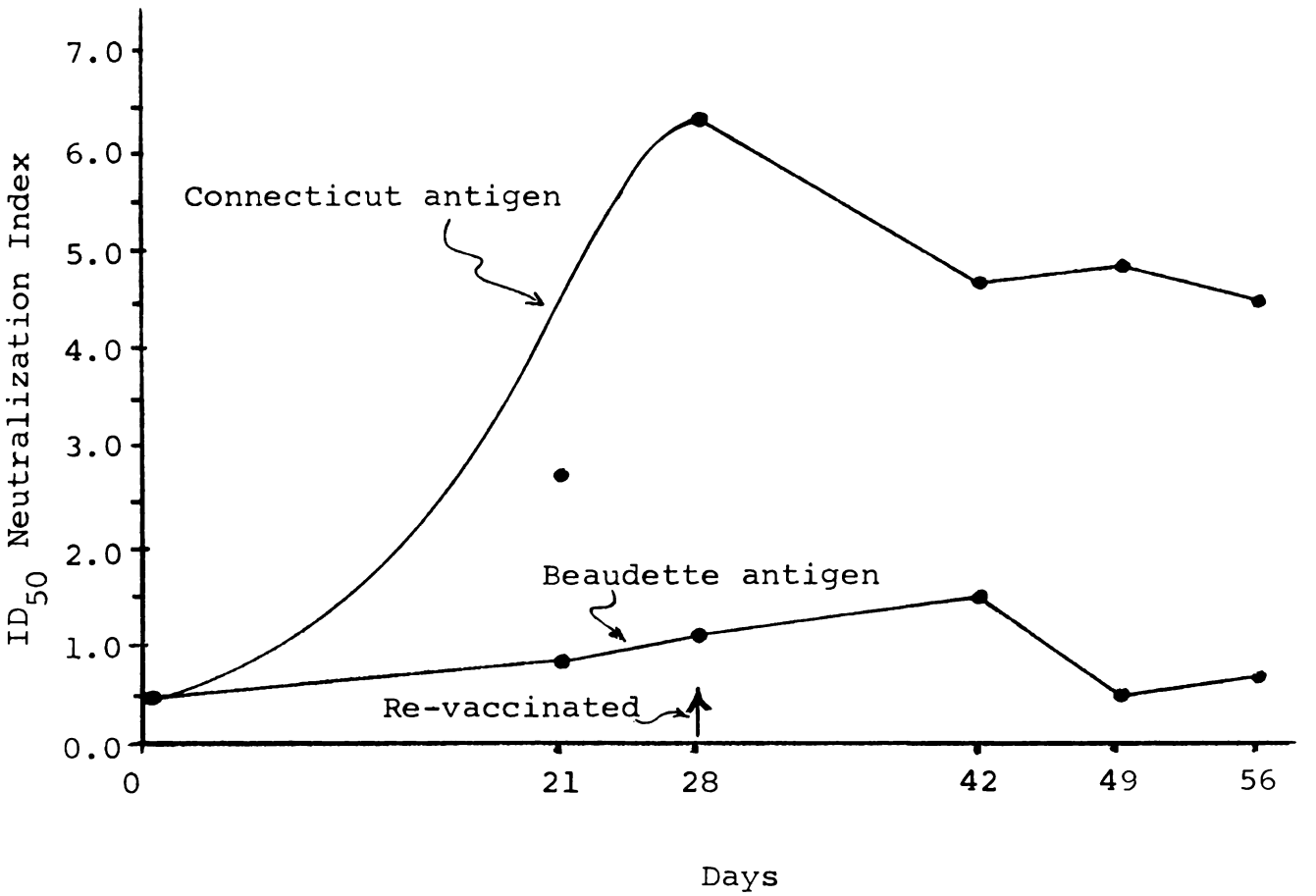


Figure 3. Neutralization tests using the Beaudette and the Connecticut antigens with serum produced in chicks (group 7A) vaccinated intranasally at 15 days of age with a commercial vaccine known to contain the Connecticut strain of IBV.

TABLE 3

Neutralization tests using the Beaudette and the Connecticut antigens with serum produced in three chickens (group 10MC) vaccinated intranasally and ocularly at 11 weeks of age with a commercial vaccine known to contain the Connecticut strain of IBV.

Accumulated days after initial vaccination		Neutralization test results	
		Beaudette antigen (V - S = NI)	Connecticut antigen (V - S = NI)
Control group 10E (7 chickens)			
(-55)	4-bird pool	6.63 - 5.17 = 1.46	. . .
(6)	2-bird pool	6.33 - 5.60 = 0.70	. . .
(62)	1-bird pool	6.60 - 5.63 = 0.97	5.50 - 4.17 = 1.33
Vaccinated group 10M-C (3 chickens)			
0	Vaccinated		
13	Re-vacc.		
27	Bled	6.33 - 6.74 = -0.41	7.0 - 0.63 = 6.3
35	Bled	5.38 - 4.83 = 0.55	7.0 - 0.22 = 6.7
36	Re-vacc.		
53	Bled	$\leq$4.63 - $\leq$3.5 = $\geq$1.1	8.22 - 0.0 = 8.22
60	Bled	. . .	. . .
68	Bled	6.60 - $\geq$5.2 = $\leq$1.4	. . .

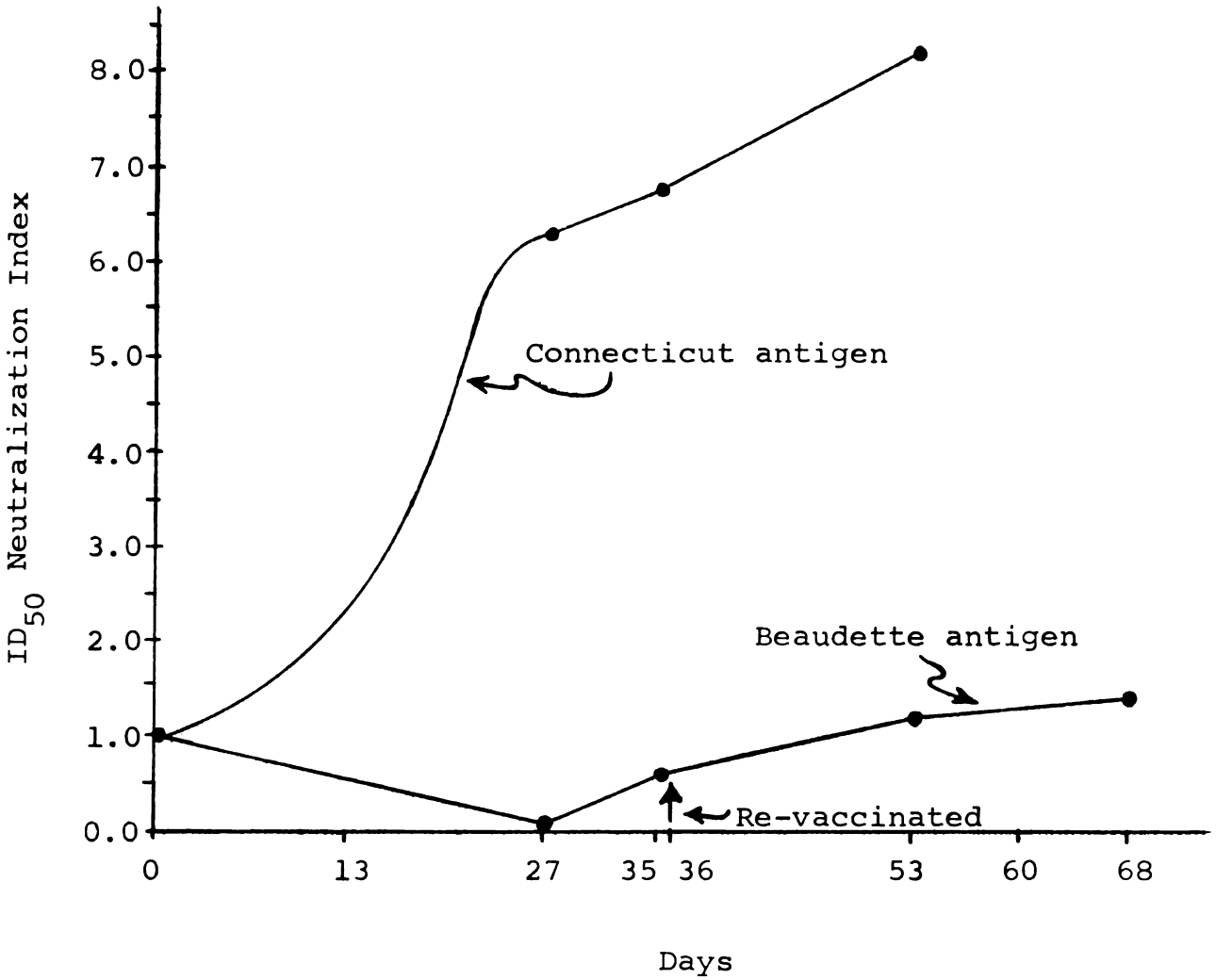


Figure 4. Neutralization tests using the Beaudette and the Connecticut antigens with serum produced in three chickens (group 10MC) vaccinated intranasally and ocularly at 11 weeks of age with a commercial vaccine known to contain the Connecticut strain of IBV.

TABLE 4

Neutralization tests using the Beaudette and the Connecticut antigens with serum produced in four chickens (group 10M-#1) inoculated intranasally and ocularly at 4 1/2 weeks of age with the Massachusetts strain of IBV.

Accumulated days after initial inoculation		Neutralization test results	
		Beaudette antigen (V - S = NI)	Connecticut antigen (V - S = NI)
Control group 10E (7 chickens)			
(-55)	4-bird pool	6.63 - 5.17 = 1.46	. . .
(6)	2-bird pool	6.33 - 5.60 = 0.70	. . .
(62)	1-bird pool	6.60 - 5.63 = 0.97	5.50 - 4.17 = 1.33
Inoculated group 10M-#1 (4 chickens)			
0	Inoculated		
13	Re-inoc.		
27	Re-inoc.		
46	Bled	6.5 - 0.83 = 5.7	7.0 - 4.2 = 2.8
55	Bled	6.33 - 1.00 = 5.33	. . .
60	Re-inoc.		
74	Bled	6.33 - 1.00 = 5.33	8.22 - 3.38 = 4.84
82	Bled	5.38 - $\leq$ 0.63 = $\geq$ 4.75	7.0 - 3.0 = 4.0
83	Re-inoc.		
100	Bled	$\leq$ 4.63 - 0.50 = $\geq$ 4.13	5.50 - 2.50 = 3.00
108	Bled	. . .	. . .
117	Bled	6.60 - 0.63 = 5.97	5.50 - 2.50 = 3.00
Pooled serum of all bleedings		6.17 - 1.00 = 5.17	5.8 - 3.75 = 2.05

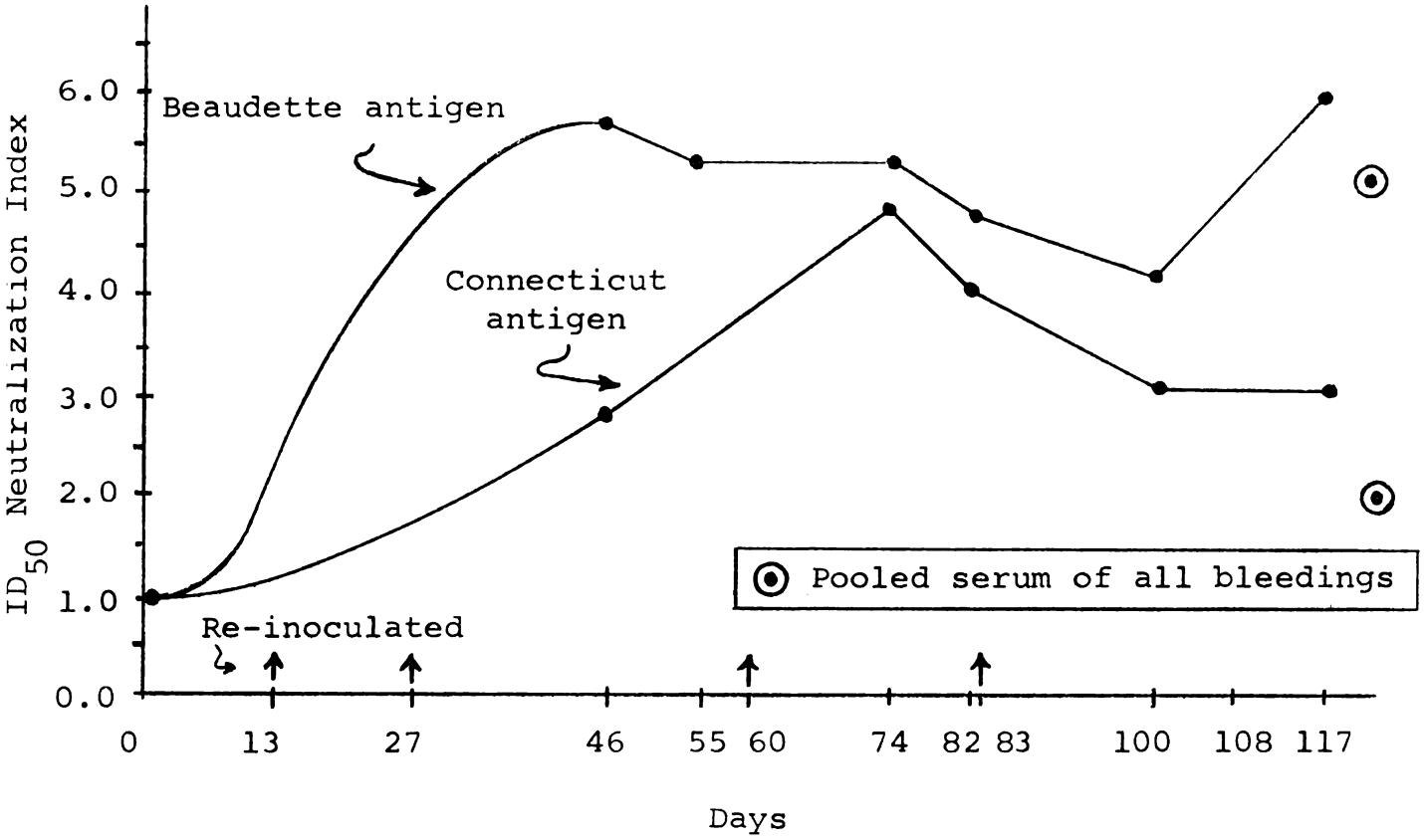


Figure 5. Neutralization tests using the Beaudette and the Connecticut antigens with serum produced in four chickens (group 10M-#1) inoculated intranasally and ocularly at 4 1/2 weeks of age with the Massachusetts strain of IBV.

TABLE 5

Neutralization tests using the Beaudette and the Connecticut antigens with serum produced in four chickens (group 10M-#2) inoculated intranasally and ocularly at 11 weeks of age with the Massachusetts strain of IBV.

Accumulated days after initial inoculation		Neutralization test results	
		Beaudette antigen (V - S = NI)	Connecticut antigen (V - S = NI)
Control group 10E (7 chickens)			
(-55)	4-bird pool	6.65 - 5.17 = 1.46	. . .
(6)	2-bird pool	6.33 - 5.60 = 0.70	. . .
(62)	1-bird pool	6.60 - 5.63 = 0.97	5.50 - 4.17 = 1.33
Inoculated group 10M-#2 (4 chickens)			
0	Inoculated		
13	Re-inoc.		
27	Bled	6.33 - 2.17 = 4.16	8.22 - 4.68 = 3.54
35	Bled	5.38 - 2.17 = 3.21	7.0 - 5.3 = 1.7
36	Re-inoc.		
53	Bled	6.75 - 0.48 = 6.27	5.50 - 2.3 = 3.20
60	Bled	. . .	. . .
69	Bled	6.60 - 0.50 = 6.10	. . .
Pooled serum of all bleedings		6.17 - 0.68 = 5.49	5.50 - 1.68 = 3.82

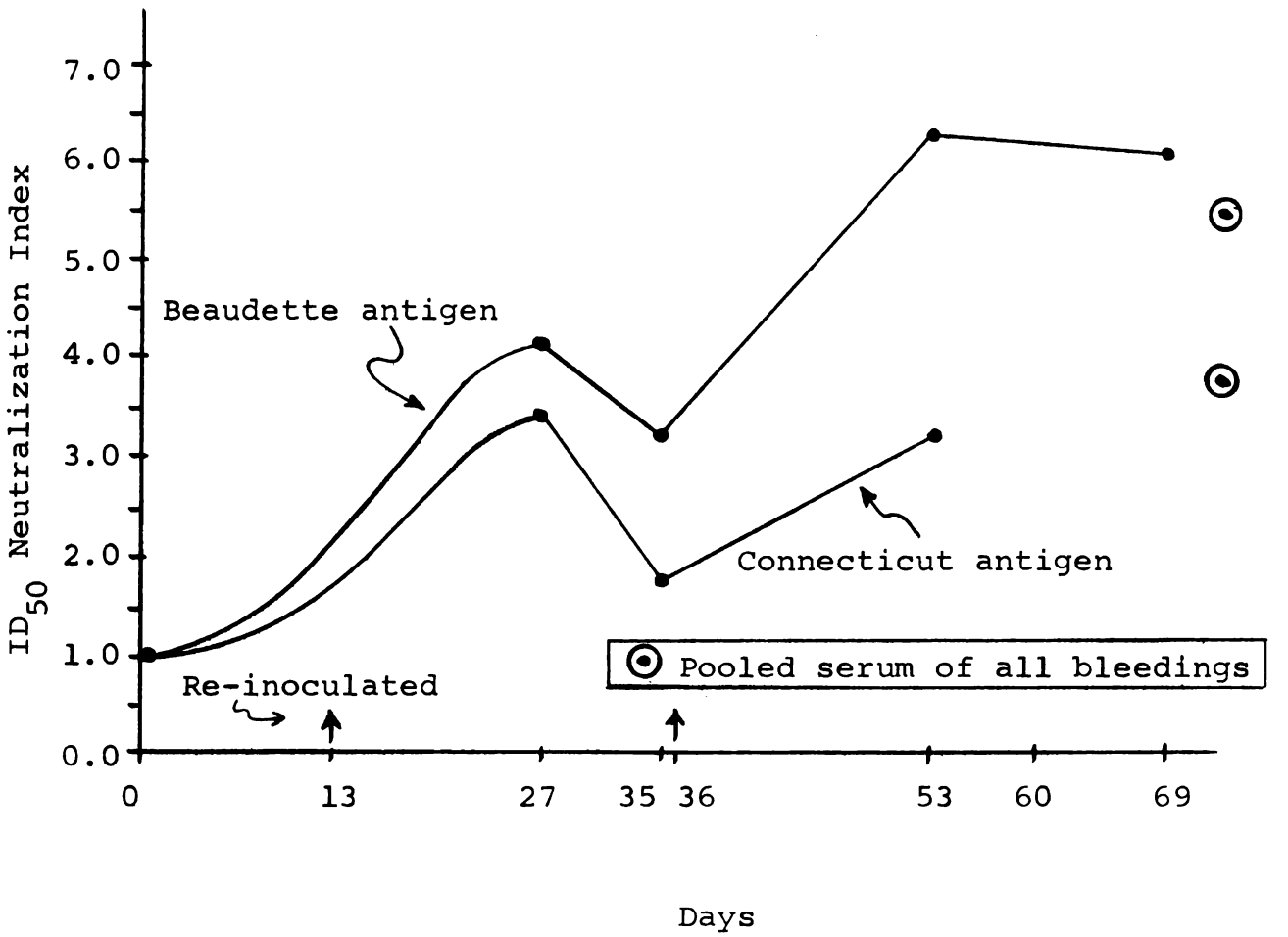


Figure 6. Neutralization tests using the Beaudette and the Connecticut antigens with serum produced in four chickens (group 10M-#2) inoculated intranasally and ocularly at 11 weeks of age with the Massachusetts strain of IBV.

TABLE 6

Time required for preparation and use of virus antigen in the neutralization test.

Minutes required	Test number								
	1	2*	3*	4	5	6*	7	8	
	Time - minutes								Av-er-age
Thaw virus from -62 C in tap water	4	4	6	3	3	4	5	3	4
Centrifuge 2000 rpm at 4 C	5	5	9	7	8	6	8	7	8
Transport to inoculating room	4	2	2	2	1	4	2	5	3
Prepare virus in 10^0 to 10^{-9} dilutions	12	16	16	11	9	10	10	10	12
Transfer dilutions to mixture tubes	14	11	10	9	10	13	12	13	11
Inoculate eggs (No. of eggs)	26 (145)	19 (150)	25 (145)	17 (70)	18 (95)	27 (135)	31 (135)	25 (110)	24 (123)
Actual incubation time of serum-virus mixtures at 4 C	26 to 48	6 to 46	15 to 40	8 to 25	12 to 32	18 to 42	12 to 44	15 to 30	14 to 38
Average incubation time	37	37	28	17	22	30	28	23	25
Actual minutes for the complete operation	84	103	76	52	58	70	71	68	72

* Tests 2, 3, and 6 above are tests 1, 2 and 3, respectively in Table 7.

The time required from thawing the antigen until the inoculated eggs were returned to the incubator ranged from 52 to 103 minutes with an average of 72 minutes when from 3 to 7 serum-virus and virus-control mixtures were used per test.

The influence of time on the titer of the Beaudette antigen at 4 C is reflected in the neutralization test results shown in Table 7 and figure 7. Nutrient broth containing antibiotics was used as the diluent. The tests indicate an average decrease in the virus-control titer of $10^{0.90}$ in 40 minutes when the virus-control titers at the beginning and the end of each of the four tests were compared.

The NI is always based on the results obtained when the virus-control mixtures were inoculated into eggs as the last step of the inoculation procedure. In all tests the NI is the reciprocal of the difference between the serum-virus titer and the virus-control titer at the end of each test (Tables 7 and 8). In test 2 of Table 7, minus neutralization indices were obtained for individual serum samples from three different control chickens. It appeared that normal avian serum might have had a greater protective influence on the infectivity of the virus than nutrient broth alone.

In later tests, nutrient broth containing antibiotics plus normal horse serum (2%) was used as the diluent (Table 8).

The reduced rate of inactivation of the Beaudette antigen is reflected by the horizontal slopes shown in Figure 8. For test 3, incubation was at room temperature (20 C) instead of 4 C. The steeper slope of inactivation shown in this one test indicates that increased temperature has a detrimental effect on the titer of the Beaudette antigen.

The virus titer in these tests was consistently higher when horse serum was contained in the diluent than when nutrient broth alone was used. Normal horse serum did not neutralize the Beaudette antigen.

TABLE 7

Influence of time on NI and virus-control titer when the Beaudette antigen is titrated at 4 C with nutrient broth as the diluent in the neutralization test for IBV.

Test	Minutes incubated at 4 C	Log_{10} dilutions										Titer	N.I.
		0	1	2	3	4	5	6	7	8	9		
No. 1**													
Virus-control	6	-	-	-	-	-	5	3	0	0	0	6.17	
10AR15-1	5	-	0	0	0	0	-	-	-	-	-	₹0.50	>4.67
Virus-control	27	-	-	-	-	-	5	1	0	0	0	5.63	
10E5-A(control)	32	-	-	-	-	5	3	0	0	-	-	5.17	0.00
10AR15-2	32	-	4	0	0	0	-	-	-	-	-	1.38	3.79
10BR17-1	38	-	-	5	5	5	2	-	-	-	-	4.83	0.34
Virus-control	46	-	-	-	-	-	3	0	2#	4#	-	₹5.17	
No. 2													
Virus-control	15	-	-	-	-	-	5	0	0	0	0	5.50	
10E14(control)	18	-	-	-	-	5	4	0	0	-	-	5.38	-0.75
10E15(control)	21	-	-	-	-	5	3	0	0	-	-	5.17	-0.54
10E17(control)	25	-	-	-	-	5	2	0*	0	-	-	4.83	-0.20
10M13-1(Mass.)	34	4	1	0	0	-	-	-	-	-	-	0.50	4.13
10MC5 (Conn.)	37	-	-	-	-	0	0	0	0	-	-	- -	- -
Virus-control	40	-	-	-	-	-	1	0	0	0	-	₹4.63	
No. 3													
Virus-control	18	-	-	-	-	-	5	5	2	0	0	6.83	
10E6 (control)	22	-	-	-	-	5	5	1	0	-	-	5.63	0.97
10MC8 (Conn.)	26	-	5	5	5	4	4	-	-	-	-	>5.25	<1.35
10M17-1(Mass.)	32	5	1	0	0	-	-	-	-	-	-	0.63	5.97
10M19-2(Mass.)	36	4*	0	0	0	0*	-	-	-	-	-	0.50	6.10
Virus-control	42	-	-	-	-	-	5	5	0	1	0*	6.60	
No. 4													
Virus-control	17	-	-	-	-	-	5	5	1	1	-	6.75	
10M2B (Mass.)	13	4	2	0	0	-	-	-	-	-	-	0.68	5.49
10M1A (Mass.)	18	3	3	1	0	-	-	-	-	-	-	1.00	5.17
11A12-1	23	4*	5	2	0	0	-	-	-	-	-	1.83	4.34
11A13-2	28	4*	4*	5	5	0	-	-	-	-	-	3.50	2.67
10M2C (Mass.)	33	4	3	0	0	-	-	-	-	-	-	1.00	5.17
Virus-control	46	-	-	-	-	-	5	3	0	0	-	6.17	

** Number and letter code identifies a specific serum.

Disregarded in calculation of virus titer due to technical error in test procedure.

* Number of positive responses per four eggs inoculated. All others are number of responses per five eggs inoculated.

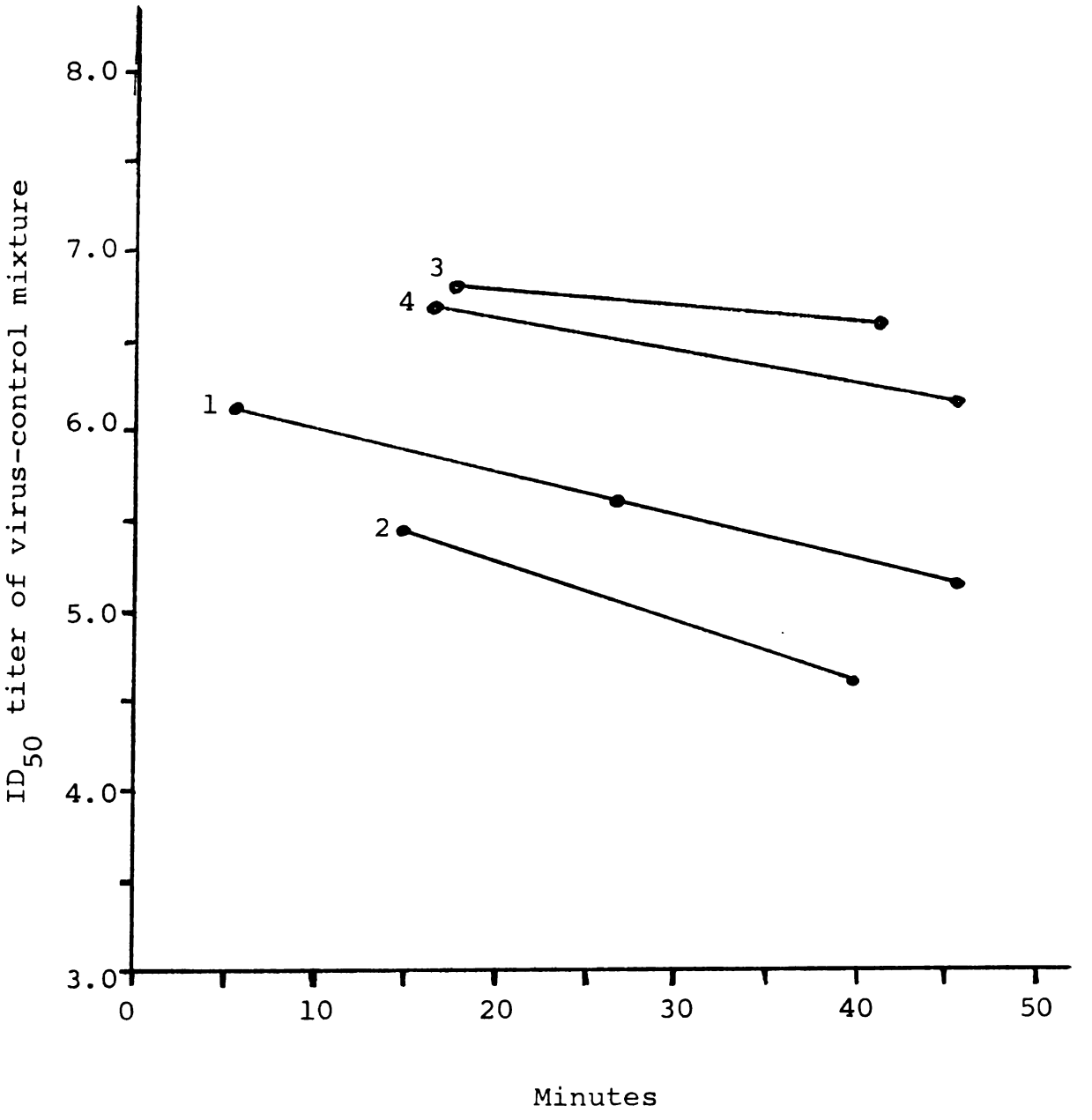


Figure 7. Influence of time on the virus-control titer when the Beaudette antigen is titrated at 4 C with nutrient broth as the diluent in the neutralization test for IBV.

TABLE 8

Influence of time on NI and virus-control titer when the Beaudette antigen is titrated at 4 C and 20 C with nutrient broth containing normal horse serum as the diluent in the neutralization test for IBV.

Test	Minutes incubated at 4 C	Log_{10} dilutions										Titer	N.I.
		0	1	2	3	4	5	6	7	8	9		
No. 1													
Virus-control	17	-	-	-	-	-	-	5	5	0*	-	7.50	
12A11-1 (test)	15	-	3	0	0	-	-	-	-	-	-	1.17	6.20
12A13 (test)	19	-	5	5	5	3#	-	-	-	-	-	>4.5	<2.8
Normal horse serum	27	-	-	-	-	-	-	5	3	0	-	7.17	0.21
Virus-control	31	-	-	-	-	-	5	5	4	0*	-	7.38	
No. 2													
Virus-control	23	-	-	-	-	-	-	5	5	1	0	7.63	
12E1 (control)	26	-	-	-	-	5	5	5	4	-	-	>7.38	<0.30
12A11-2 (test)	26	-	3	0	0	0	-	-	-	-	-	1.17	6.51
12A13 (test)	34	-	5	5	5	4	0*	-	-	-	-	4.38	3.30
12A6 (test)	40	-	5	5	5	5	2	-	-	-	-	4.83	2.85
12A15-3 (test)	46	-	4	5	5	1	2#	-	-	-	-	3.75	3.93
Virus-control	57	-	-	-	-	-	4*	5	4	2	0#	7.68	
No. 3	(20 C)												
Virus-control	16	-	-	-	-	-	-	5	5	2	0	7.83	
TPV-4 (turkey)	14	-	-	-	5	5	5	5	-	-	-	>6.5	<0.88
TPV-5 (turkey)	21	-	-	-	-	5	5	5	5	-	-	>7.5	-0.12
TPV-6 (turkey)	25	-	-	-	-	4*	5	5	4	-	-	>7.38	<0.00
12A17-1 (test)	21	-	3	1	0	0	0	-	-	-	-	1.32	6.06
12A18-1 (test)	23	-	1	1	0	0	-	-	-	-	-	<0.75	>6.63
Virus-control	43	-	-	-	-	-	5	5	4	0	0	7.38	
No. 4													
Virus-control	23	-	-	-	-	-	-	4*	3	0	0	7.17	
TPV-1 (turkey)	24	-	-	-	-	-	4*	5	0	-	-	6.50	1.00
12A24-1 (test)	21	-	5	4	0	0	-	-	-	-	-	2.38	5.12
12E4-2 (control)	32	-	-	-	-	-	5	5	3	-	-	>7.17	<0.33
12E6-1 (control)	35	-	-	-	-	5	4*	4*	2	-	-	>6.83	<0.67
12E8-1 (control)	40	-	-	-	-	5	5	5	3*	-	-	>7.33	<0.17
Virus-control	47	-	-	-	-	-	-	5	4	1	0	7.50	

(Continued on next page)

TABLE 8 (Continued)

Test	Minutes incubated at 4 C	Log_{10} dilutions										Titer	N.I.
		0	1	2	3	4	5	6	7	8	9		
No. 5													
Virus-control	16	-	-	-	-	-	-	4*	5	1	0	7.63	
12A5 (test)	17	-	-	5	5	3	1	1*	-	-	-	4.46	3.37
12A16 (test)	20	-	-	5	2	2	1	-	-	-	-	3.41	4.42
12A22 (test)	25	-	4*	5	1	2	0	-	-	-	-	2.88	4.95
12A29 (test)	30	-	4*	5	5	5	2*	-	-	-	-	>5.00	<2.83
Virus-control	37	-	-	-	-	-	5	5	5	2	0	7.83	

* Number of positive responses per four eggs inoculated.

Number of positive responses per three eggs inoculated.

All others are number of positive responses per five eggs inoculated and are used in calculating viral activity.

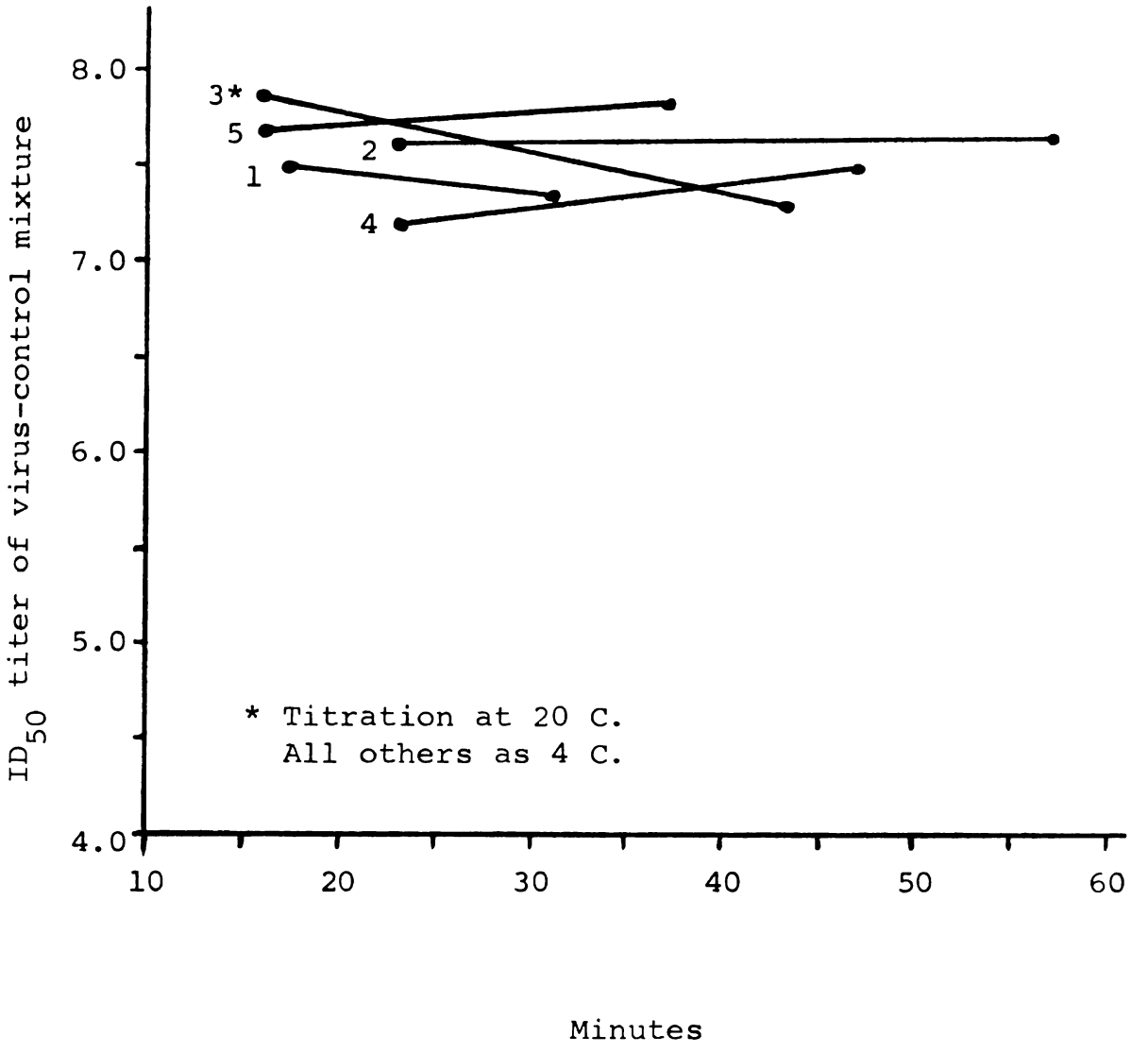


Figure 8. Influence of time on the virus-control titer when the Beaudette antigen is titrated at 4 C and 20 C with nutrient broth containing normal horse serum as the diluent in the neutralization test for IBV.

B. Serology

Different methods in collecting, pooling, processing, and storing serum samples were examined as possible variables in the neutralization test for IBV using the Beaudette antigen.

In Tables 9 to 13, both the serum-virus titer and the virus-control titer are shown to indicate the effect of a virus with either a high or a low titer on the NI. In most instances the paired serum samples were tested on the same day to equalize differences due to variation of the virus titer from one test to the next.

The data in Tables 10 to 13 are also presented in figures 10 to 13 as scatter diagrams. Each point on the diagram represents the neutralization indices obtained from two serum

samples paired for comparison. The dotted line extending upward at 45° from the point of origin represents the "line of equality for paired observations" upon which all points would have been plotted if no differences in neutralization indices were found. The preponderance of points plotted upon one side of this line indicates a significant change in the neutralization indices toward that direction. The perpendicular distance from any point to the line of equality is equal to the difference in paired observations used to obtain that point.

1. Neutralization indices of individual serum and pooled serum of the same two chickens.

When two individual anti-IBV serums are pooled in equal portions, the neutralization index of each serum loses its identity.

For example, the difference between neutralization indices for ten pairs of individual serum samples ranged from 0.15 to 2.77 (Table 9, figure 9).

When the antilogs of the NI of two individual anti-IBV serums were averaged, the log of the average expressed as the expected NI was found to be within $\pm$ 0.3 log unit of the observed NI for the pooled serum of the two individual serums in 7 of 10 (70%) tests. Of the other three tests, the observed NI of the pooled serum was higher than the calculated average in two tests and lower in one test.

TABLE 9

Neutralization indices of individual serum and pooled serum of the same two chickens.

Pool No.	Serum sample	Pooled serum (A + B) (V - S = NI)	Individual serum (V - S = NI)	Difference between NI of A and B
1	2B5	5.50 - 2.33 = 3.17	A 5.50 - 4.67 = 0.83 B 5.50 - <2.00 = >3.50	2.67
2	3D2	6.67 - 5.50 = 1.17	A 6.67 - 4.50 = 2.17 B 6.67 - >6.38 = <0.29	1.88
3	4A3	6.63 - 5.32 = 1.31	A 6.63 - 5.50 = 1.13 B 6.63 - 4.84 = 1.79	0.66
4	4C9	7.32 - 5.50 = 1.82	A 7.32 - 5.50 = 1.82 B 7.32 - 5.78 = 1.52	0.30
5	5B1	5.67 - 4.50 = 1.17	A 5.67 - 4.38 = 1.29 B 6.83# - 4.45 = 2.38	1.09
6	6A5	6.83 - 4.50 = 2.37	A 6.83 - 4.48 = 2.35 B 6.83 - 5.63 = 1.20	1.15
7*	6A7	6.17 - 3.00 = 3.17	A 6.17 - 2.63 = 3.54 B 6.17 - 4.63 = 1.54	2.00
8	9A29	6.00 - 1.32 = 4.68	A 6.00 - 3.78 = 2.22 B 6.31# - 1.32 = 4.99	2.77
9	9A33	6.17 - 1.46 = 4.71	A 6.17 - 3.32 = 2.85 B 6.17 - 1.32 = 4.85	2.00
10	9A35	6.63 - <0.63 = >6.00	A 6.63 - 1.32 = 5.31 B 6.63 - 1.17 = 5.46	0.15

* Serum subjected to 56 C for 30 minutes. All other serum not heated.

\# Test performed on different day from other tests of this serum.

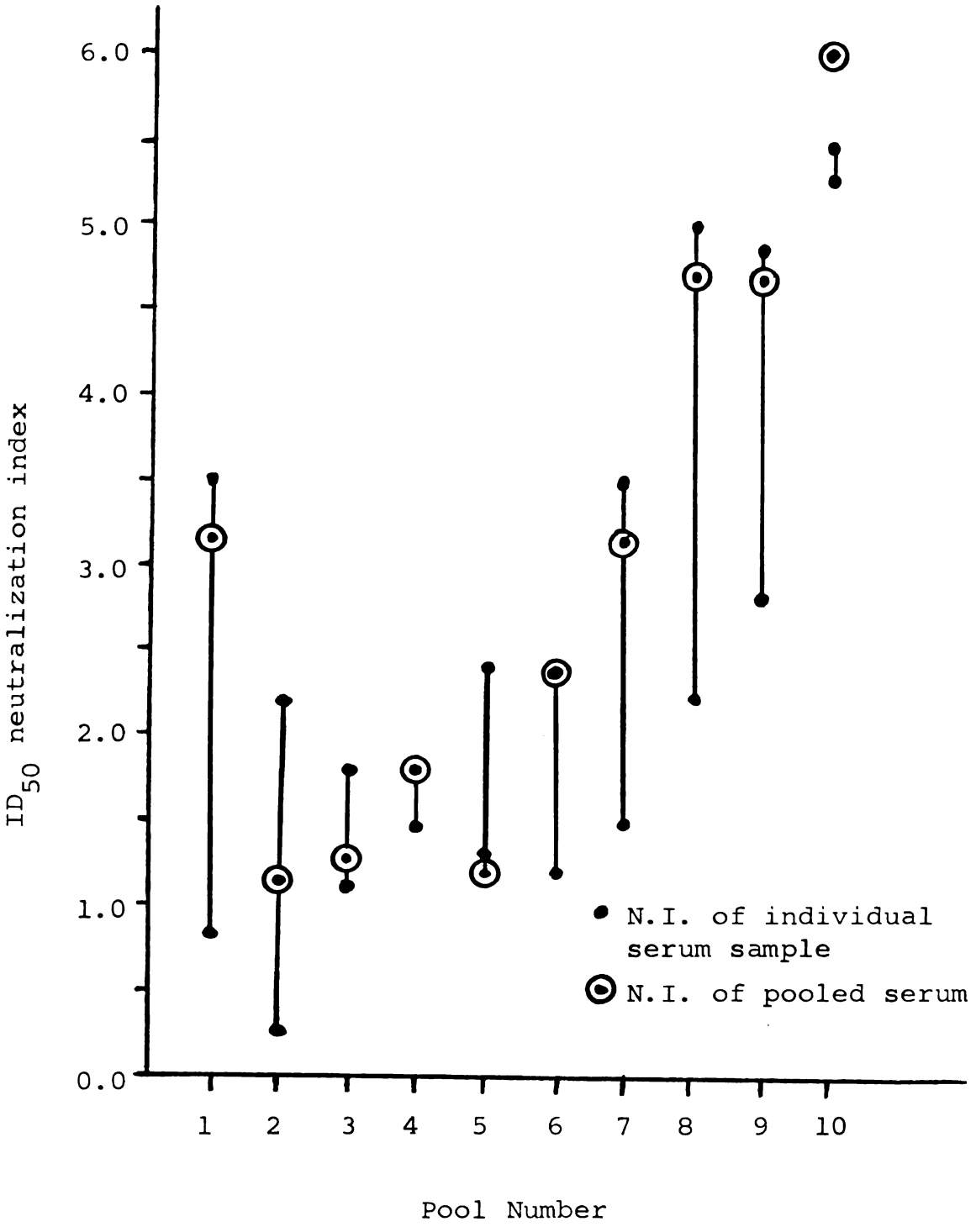


Figure 9. Neutralization indices of individual serum and pooled serum of the same two chickens.

A minimum NI of 3.0 is the critical value for assay of immunity induced by IB vaccines. In this study only 6 of 20 (30%) individual serums had a NI above 3.0. With the pool of two individual serums, 5 of 10 (50%) pools had a NI above 3.0. Of these five pools, four contained an individual serum with a NI below 3.0 that was obscured by its companion serum.

2. Neutralization indices of serum collected from the same chicken(s) 21 and 28 days after a single intranasal administration of different serials of IB vaccines to 15-day old chicks.

The neutralization indices of serum collected from the same chicken 21 and 28 days after vaccination are shown in Table 10 and plotted in figure 10 with the "line of equality for paired observations." Of the 23 paired observations, 21 occurred on the 28-day side of the line. The differences in the neutralization indices range from -0.35 to 1.74 for serum from the same chicken or pair of chickens if the serums were pooled.

Of the 21 serums collected 21 days after vaccination, 14 (61%) had a NI of 3.0 or above. For serums from the same chickens collected 28 days after vaccination, 18 (78%) had a NI of 3.0 or above.

This study indicates that the NI for chickens bled 28 days after a single vaccination is significantly higher than the NI for the same chickens bled 21 days after vaccination.

For a statistical test of the significant difference between paired observations (Dixon and Massey, 1957) let x_i and

y_i represent the NI of serum collected from the ith chicken (or the ith pair of chickens if the serums were pooled) 21 and 28 days, respectively, after vaccination (Table 10).

The difference (d_i) between x_i and y_i is represented by $d_i = y_i - x_i$, where $i = 1,2,3,\cdots,n$ observations. The mean and the standard deviation of these differences are represented by $\bar{d}$ and s_d, respectively.

The mean difference is calculated from $\bar{d} = \frac{\Sigma d_i}{n}$; and the standard deviation from $s_d = \sqrt{\frac{\Sigma(d_i^2) - \frac{(\Sigma d_i)^2}{n}}{n-1}}$.

The following data are obtained from Table 10: $n = 23$; $\Sigma d_i = 16.52$; $\Sigma(d_i^2) = 18.5099$; $(\Sigma d_i)^2 = 272.91$. Thus, the mean difference ($\bar{d}$) is .718, and the standard deviation of the mean (s_d) is .5495.

It is assumed that the differences (d_i) have a normal distribution with the population mean (μ_d). Under the null hypothesis, $\mu_y - \mu_x = \mu_d = 0$. That is, for all chickens in a population the mean difference in the neutralization indices for serum collected at 28 and 21 days after a single vaccination is zero.

The alternate hypothesis is that $\mu_y - \mu_x = \mu_d > 0$. That is, the mean difference in the neutralization indices is greater on the 28th day than on the 21st day after vaccination.

Under the null hypothesis that $\mu_d = 0$, the statistic $t = \frac{\bar{d}}{s_d / \sqrt{n}}$ has a t-distribution with (n-1) degrees of freedom. At the 5% level of significance with a one-sided test, the null hypothesis would be rejected if $t > t_{(.05)}(22) = 1.72$. In this case, $t = \frac{.718}{(.5495) / \sqrt{23}} = 6.26$, so the null hypothesis is rejected. The conclusion is that the NI for serum collected 28 days after a single vaccination is significantly higher at the 5% level of significance than for serum collected from the same chicken 21 days after vaccination.

The 90% confidence interval for the mean difference in neutralization indices is calculated from:

$$\bar{d} - \frac{(s_d)t_{(.05)}(22)}{\sqrt{n}} < \mu_d < \bar{d} + \frac{(s_d)t_{(.05)}(22)}{\sqrt{n}}$$

The 90% confidence interval is $.480 < \mu_d < .956$, or that there is 90% confidence that the true mean difference (μ_d) for serum collected 28 days after vaccination lies between 0.5 and 0.9 log units greater than the NI obtained from serum collected from the same chicken 21 days after a single vaccination against IBV.

The data presented in Table 10 are subject to some criticism as the serums used for most of the paired observations were not subjected to the same conditions when tested. First, the serums collected on the 21st and 28th days were usually

not tested on the same day. Second, some serums were subjected to 56 C for 30 minutes and their duplicate sample was not. Third, some serum was stored at -27 C while its duplicate sample was stored at 4 C, and fourth, clearly defined end-points were not obtained in all tests. Each of these conditions obscures the individual findings, but when used together and analyzed, a definite pattern of change in the neutralization indices was observed.

3. Neutralization indices after various methods of processing and storing serum samples.

The neutralization indices from duplicate serum samples where one sample was subjected to 56 C for 30 minutes, the other one non-heated, then both stored at either 4 C (Table 11) or at -27 C (Table 12) were compared. These respective data are presented in figure 11 as a scatter diagram in which all 7 paired observations are on the side of the "line of equality for paired observations" for non-heated serum. In figure 12, all 9 paired observations are on the side of the line of equality for non-heated serum.

In Table 11, the range of differences of the neutralization indices was from 0.50 to 1.93 for serums stored at 4 C. In Table 12 the range of differences was from 0.32 to 1.49 for serums stored at -27 C.

TABLE 10

Neutralization indices of serum collected from the same chicken(s) 21 and 28 days after a single intranasal administration of different serials of various IB vaccines to 15-day old chickens.

21 day bleeding (x_i)	28 day bleeding (y_i)	Difference in NI (d_i)
7.56 - 2.17 = 5.4	7.17 - <0.83 = >6.34	0.94
♀7.56 - 3.75 = 3.8	6.67 - <1.5 = >5.17	1.37
#7.4 - 4.17 = 3.2	#6.22 - 2.38 = 3.84	0.64
7.4 - 3.50 = 3.9	6.22 - 2.20 = 4.02	0.12
7.4 - 5.50 = 1.9	6.00 - 4.32 = 1.68	-0.22
#7.4 - 2.38 = 4.0	#7.37 - 1.63 = 5.74	1.74
#7.4 - 4.68 = 2.7	#7.37 - 3.17 = 4.20	1.50
7.4 - 2.7 = 4.8	6.31 - 1.32 = 5.00	0.20
7.4 - 5.38 = 2.0	6.00 - 3.78 = 2.22	0.22
7.4 - 2.6 = 4.8	6.84 - 1.33 = 5.51	0.71
7.4 - 3.53 = 3.9	6.84 - 1.83 = 5.01	1.11
7.4 - 4.17 = 3.2	6.17 - 3.32 = 2.85	-0.35
6.31 - 2.52 = 3.8	6.17 - 1.50 = 4.7	0.90
6.31 - <2.6 = >3.7	6.17 - 1.68 = 4.49	0.79
6.31 - <2.6 = >3.7	6.17 - 2.38 = 3.8	0.10
6.31 - <1.5 = >4.8	6.63 - <0.63 = >6.0	1.20
¡*6.68 - 4.22 = 2.46	6.68 - 3.75 = 2.93	0.47
¡*7.68 - 3.50 = 4.18	7.68 - 2.3 = 5.38	1.20
¡*5.43 - 2.5 = 2.93	#5.43 - 2.0 = 3.43	0.50
¡ 6.83 - 4.50 = 2.33	#6.17 - 3.0 = 3.17	0.84
¡ 6.83 - 4.48 = 2.35	#6.17 - 2.63 = 3.54	1.19
¡ 6.83 - 5.63 = 1.20	#6.17 - 4.63 = 1.54	0.34
¡*6.00 - 3.33 = 2.67	#6.00 - 2.32 = 3.68	1.01

* 21 and 28 day bleedings tested same day; all others not tested same day.

\# Serum subjected to 56 C for 30 minutes; all others non-heated.

¡ Serums from chickens which had received IB vaccine.

♀ Serums from chickens which had received Newcastle-Bronchitis vaccine.

All other serums from chickens which had received Newcastle-Bronchitis-Laryngotracheitis vaccine.

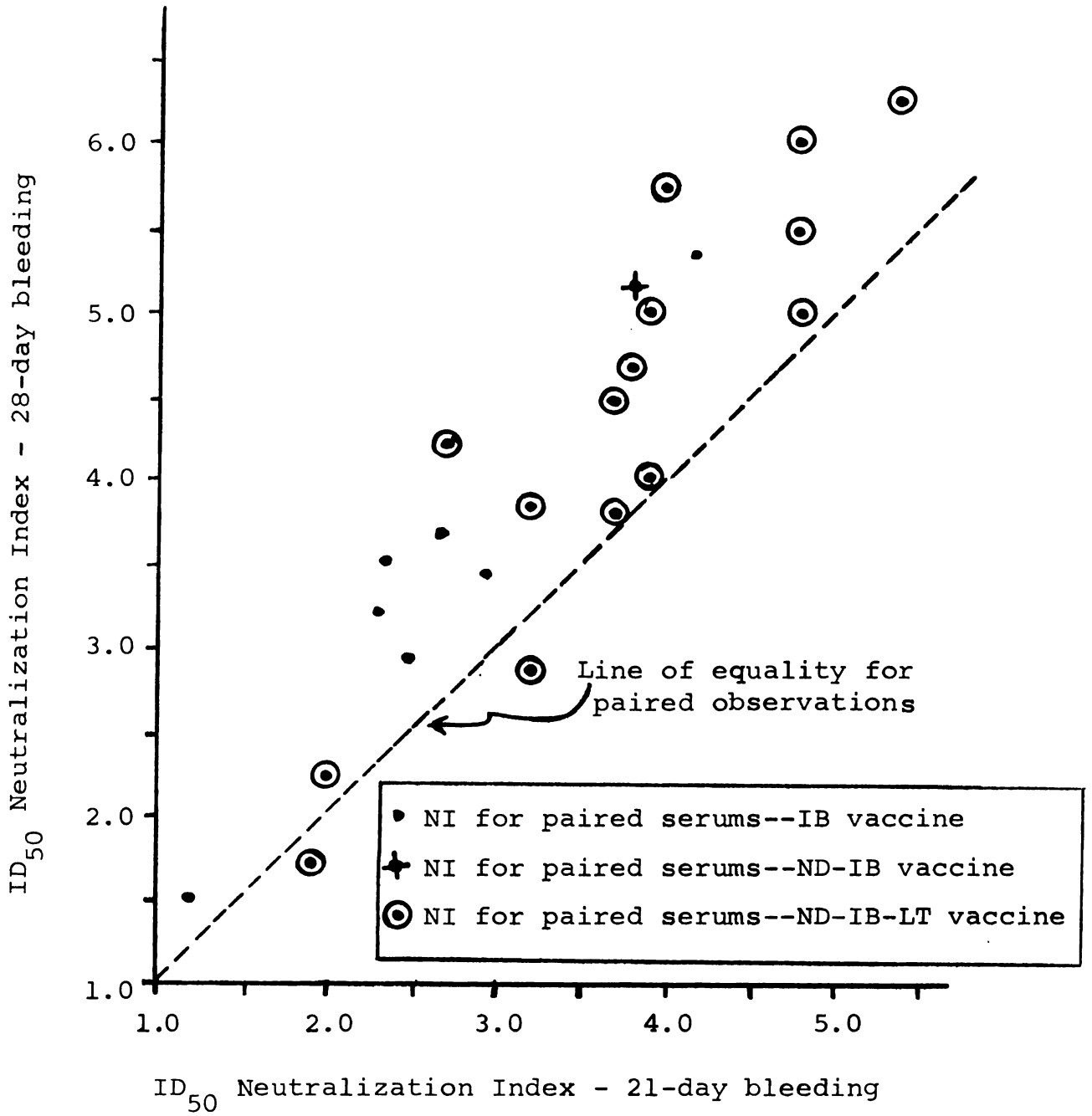


Figure 10. Neutralization indices of serum collected from the same chicken(s) 21 and 28 days after a single intranasal administration of different serials of various IB vaccines to 15-day old chickens.

TABLE 11

Neutralization indices from duplicate serum samples where one serum was subjected to 56 C for 30 minutes, and the other non-heated, and both samples stored at 4 C.

Heated serum (x_i)	Non-heated serum (y_i)	Difference in NI (d_i)
6.67 - 5.50 = 1.17	6.67 - 4.83 = 1.84	0.67
7.17 - 4.00 = 3.17	7.17 - 3.50 = 3.67	0.50
*7.17 - 4.22 = 2.95	7.17 - 3.68 = 3.49	0.54
*7.17 - >6.17 = <1.0	7.17 - 5.32 = 1.85	0.85
*5.63 - 4.66 = 0.97	5.63 - 3.3 = 2.3	1.33
*5.63 - 4.63 = 1.0	5.63 - <2.7 = >2.9	>1.90
#<5.75 - 3.32 = <2.43	6.83 - 2.50 = 4.33	>1.93

\# Not tested on same day as duplicate sample.

* Serum from chickens which had received Newcastle-Bronchitis vaccine.

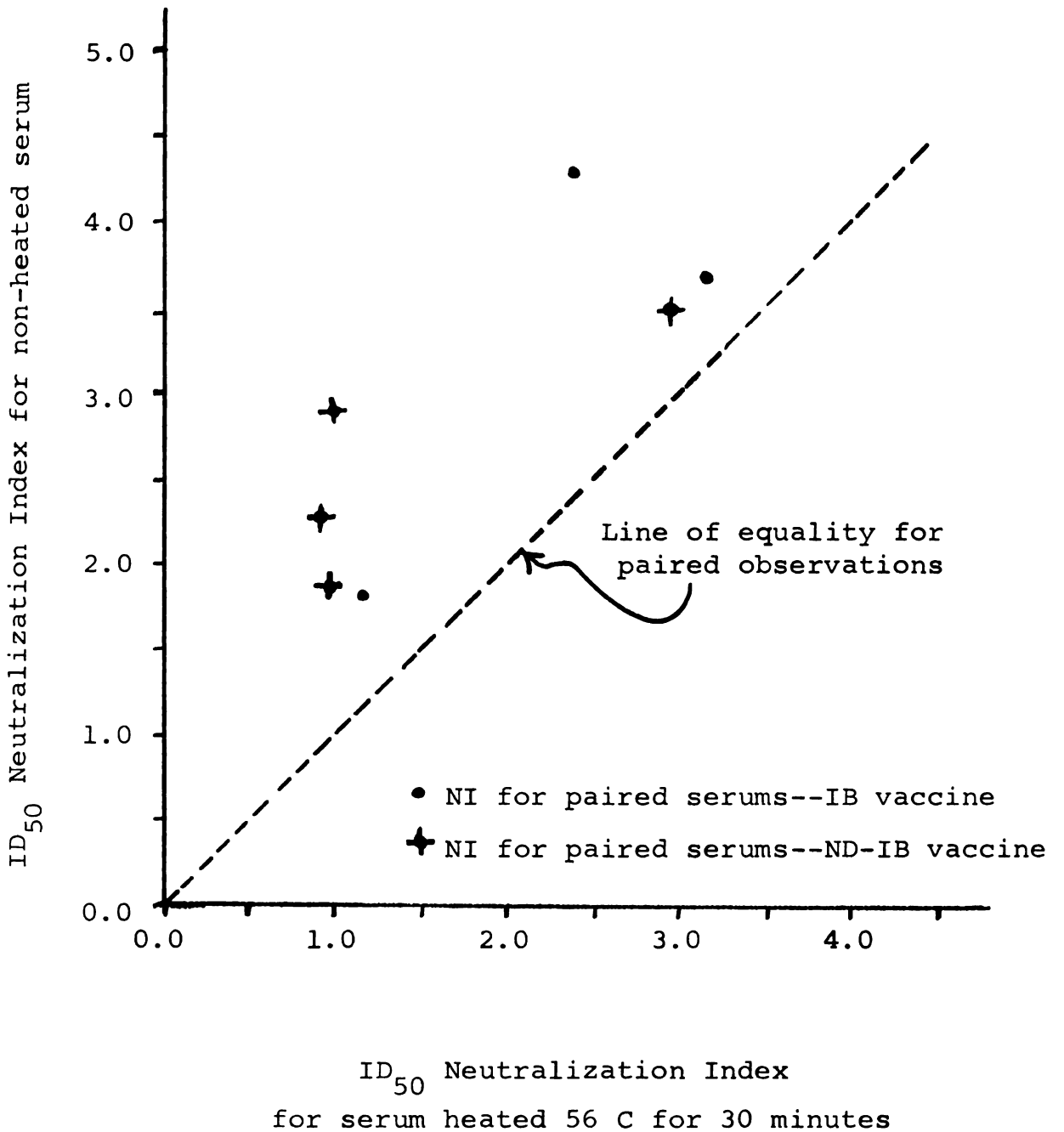


Figure 11. Neutralization indices from duplicate serum samples where one serum was subjected to 56 C for 30 minutes, the other non-heated, and both samples stored at 4 C.

TABLE 12

Neutralization indices from duplicate serum samples where one serum was subjected to 56 C for 30 minutes, the other non-heated, and both samples stored at -27 C.

Heated serum (x_i)	Non-heated serum (y_i)	Difference in NI (d_i)
#6.67 - 2.50 = 4.17	6.67 - 1.5 = 5.17	1.00
6.22 - 2.38 = 3.84	6.22 - 1.63 = 4.59	0.75
6.22 - 3.32 = 2.90	6.22 - 2.20 = 4.02	1.12
6.22 - 5.17 = 1.05	*6.00 - 4.32 = 1.68	0.63
7.37 - 1.63 = 5.74	7.37 - 1.17 = 6.20	0.46
7.37 - 3.17 = 4.20	7.37 - 2.63 = 4.74	0.54
6.84 - 2.17 = 4.67	6.84 - 1.33 = 5.51	0.84
6.84 - 3.32 = 3.52	6.84 - 1.83 = 5.01	1.49
6.17 - 2.00 = 4.17	6.17 - 1.68 = 4.49	0.32

* Not tested on same day as duplicate sample.

\# Paired serums from chickens which had received Newcastle-Bronchitis vaccine. All other serums from chickens which had received combined Newcastle-Bronchitis-Laryngotracheitis vaccine.

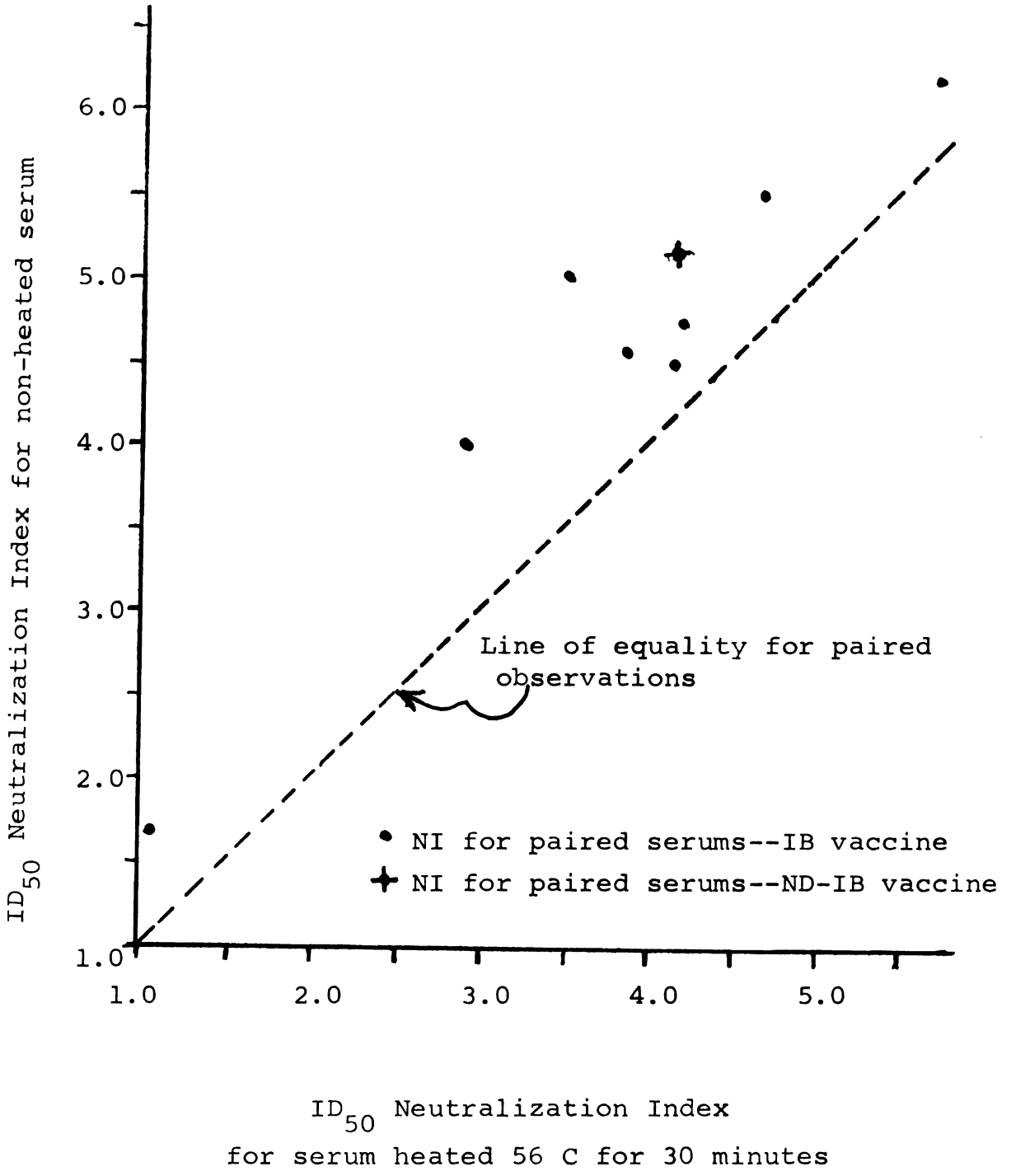


Figure 12. Neutralization indices from duplicate serum samples where one serum was subjected to 56 C for 30 minutes, the other non-heated, and both samples stored at -27 C.

The conclusion from these data is that serum subjected to 56 C for 30 minutes and stored at either 4 C or -27 C has a significantly lower NI than non-heated serum. This suggests the possibility of non-specific factors being present in avian serum that may partially neutralize IBV.

From the data in Table 11, the following:

$n = 7$; $\Sigma d_i = 7.72$; $\bar{d} = 1.10$; $\Sigma(d_i^2) = 10.8170$;

$(\Sigma d_i)^2 = 59.60$; $s_d = .619$; $t_{(.05)}(6) = 1.94$;

$t = \frac{\bar{d}}{s_d / \sqrt{n}} = 4.7$, may be used for the same statistical analysis as used for the data in Table 10.

From the above data, the null hypothesis that the mean difference (μ_d) is zero is rejected at the 5% level of significance with a one-sided test. The conclusion is that the NI for heated serum is significantly lower than the NI for non-heated serum when both are stored at 4 C.

There is 90% confidence that the true mean difference of the NI for paired observations from heated and non-heated serum would be between .53 and 1.67 log units lower for heated serum than for non-heated serum stored at 4 C.

From the data in Table 12, the following:

$n = 9$; $\Sigma d_i = 7.15$; $\Sigma(d_i^2) = 6.7466$; $\bar{d} = .794$;

$(\Sigma d_i)^2 = 51.12$; $s_d = .364$; $t_{(.05)}(8) = 1.86$;

$t = \frac{\bar{d}}{s_d / \sqrt{n}} = 6.24$, may be used for the same statistical analysis as used for the data in Table 10.

From the above data, the null hypothesis that the mean difference (μ_d) is zero is rejected at the 5% level of significance with a one-sided test. The conclusion is that the NI for heated serum is significantly lower than the NI for non-heated serum when both are stored at -27 C.

There is 90% confidence that the true mean difference of the NI for paired observations from heated and non-heated serum would be between .514 and 1.07 log units lower for heated serum than for non-heated serum stored at -27 C.

The neutralization indices from duplicate serum samples where one sample was stored at 4 C and the other one at -27 C (Table 13) were compared. The samples stored at 4 C were divided into two periods of storage: one of less than 4 weeks, and the other of from 7 to 36 weeks. The frozen and non-frozen samples were tested on the same day except as noted in Table 13.

In Table 13, the range of the differences of the neutralization indices was from -0.62 to 1.13 log units. These data are presented in figure 13 as a scatter diagram in which 8 of 13 paired observations occur on the side of the "line of equality for paired observations" for serum stored at -27 C. However, the short perpendicular distance from any plotted point to the line of equality indicates that the differences in the paired observations are not great.

From Table 13, three groups of data are assembled for the same statistical analysis used for the data in Table 10:

For serums stored less than 4 weeks at 4 C compared with serums stored at -27 C:

$n = 4$; $\Sigma d_i = 2.36$; $\Sigma(d_i^2) = 2.1004$; $\bar{d} = .59$;

$(\Sigma d_i)^2 = 5.5696$; $s_d = .485$; $t_{(.05)}(3) = 2.353$;

$$t = \frac{\bar{d}}{s_d / \sqrt{n}} = 2.43.$$

For serums stored from 7 to 36 weeks at 4 C compared with serums stored at -27 C:

$n = 9$; $\Sigma d_i = 1.62$; $\Sigma(d_i^2) = 1.8326$; $\bar{d} = .18$; $(\Sigma d_i)^2 = 2.6244$;

$s_d = .437$; $t_{(.05)}(8) = 1.86$; $t = \frac{\bar{d}}{s_d / \sqrt{n}} = 1.235.$

For the overall totals for serum stored at 4 C compared with serum stored at -27 C:

$n = 13$; $\Sigma d_i = 3.98$; $\Sigma(d_i^2) = 3.924$; $\bar{d} = 3.924$;

$(\Sigma d_i)^2 = 15.8404$; $s_d = .377$;

$t_{(.05)}(12) = 1.78$; $t = \frac{\bar{d}}{s_d / \sqrt{n}} = 2.81$

For the small sample size of 4 used in the statistical analysis for serum stored at 4 C for less than 4 weeks, the null hypothesis of no significant difference is rejected at the 5% level of significance with a one-sided test.

TABLE 13

Neutralization indices of duplicate serums where one serum was stored at 4 C and the other serum at -27 C.

Days at 4 C	Storage at 4 C (x_i)	Storage at -27 C (y_i)	Difference in NI (d_i)
Stored less than 4 weeks.			
14	#6.50 - 4.39 = 2.11	6.50 - 3.26 = 3.24	1.13
15	7.00 - 3.63 = 3.37	7.00 - 3.68 = 3.32	-0.05
15	7.00 - 3.84 = 3.16	7.00 - 3.17 = 3.83	0.67
26*	#6.83 - 6.25 = 0.58	6.83 - 5.54 = 1.29	0.61
Stored 7 to 36 weeks.			
85	7.17 - 2.50 = 4.67	7.17 - 2.50 = 4.67	0.00
85*	7.17 - 3.32 = 3.85	7.17 - 3.32 = 3.85	0.00
89*	6.75 - 3.00 = 3.75	6.75 - 2.32 = 4.43	0.68
110	#5.43 - 2.00 = 3.43	5.43 - <1.63 = >3.80	>0.40
117	5.43 - 2.50 = 2.93	5.43 - 2.38 = 3.05	0.12
117	7.68 - 1.68 = 6.00	7.68 - 2.30 = 5.38	-0.62
152	8.00 - 3.32 = 4.68	8.00 - 2.50 = 5.50	0.82
208	6.68 - 2.17 = 4.51	6.68 - 1.83 = 4.85	0.34
251	6.50 - 3.38 = 3.12	6.50 - 3.50 = 3.00	-0.12

\# Both paired samples subjected to 56 C for 30 minutes before storage.

* Paired serums from chickens which had received Newcastle-Bronchitis vaccine. All other serums from chickens which had received IB vaccine.

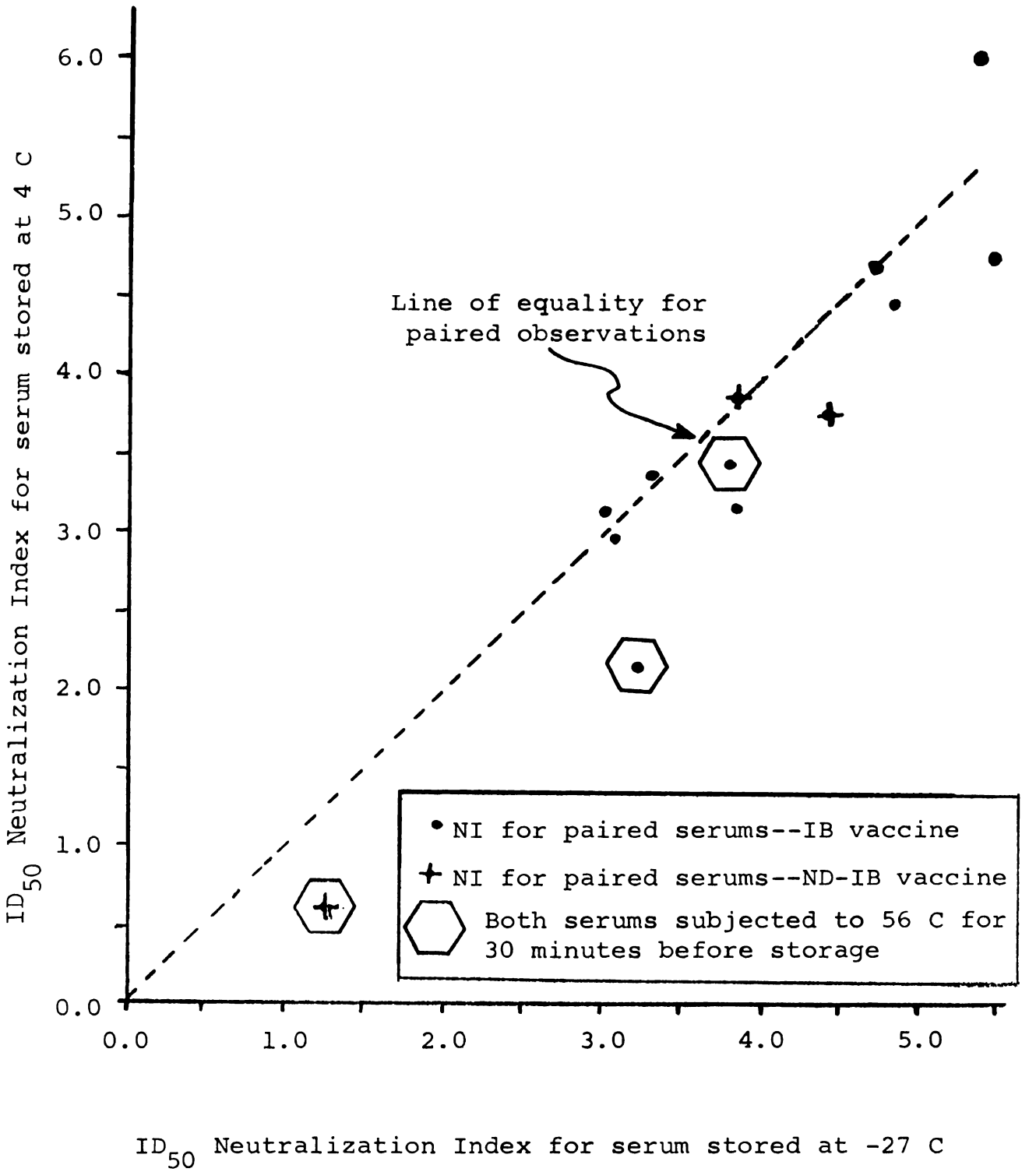


Figure 13. Neutralization indices of duplicate serums where one serum was stored at 4 C and the other serum at -27 C.

With the sample size of 9 used for serum stored from 7 to 36 weeks, the null hypothesis that the mean difference (μ_d) is zero cannot be rejected at the 5% level of significance with a one-sided test.

When all observations in Table 13 are used in statistical analysis, the sample size of 13 indicates that the null hypothesis of no significant difference between the paired observations is rejected at the 5% level of significance with a one-sided test.

However, when the 90% confidence limits for the mean difference (μ_d) is calculated, it varies only from 0.07 to 0.54 log units in favor of serum stored at -27 C.

C. Histopathology

Evaluation of changes in the tracheal mucosa following intranasal administration of an IB vaccine was undertaken as another possible method for critical study of the immunizing ability of commercial vaccines.

The graded response of the tracheal mucosa following a single intranasal vaccination is shown in figure 14 for eight groups of chickens, each of which received a vaccine from a different manufacturer.

The normal mucosa of 15- and 36-day old chicks shown in figures 17 and 18 bears pseudostratified ciliated epithelium with goblet cells and mucous crypts.

Following vaccination, the mucosa underwent cyclic changes in three main phases with a return to approximately normal in about 21 days: (1) acute phase (3 to 9 days), (2) reparative phase (9 to 15 days), and (3) immune phase (15 to 21 days). The tracheae from group 7A (figures 19 to 26) were used to illustrate a relatively typical response at three-day intervals following vaccination.

With the five groups (6A, 6B, 7A, 7B and 7C) receiving the single component IB vaccine, the maximum of the acute phase was reached on the sixth to ninth day where there was epithelial hypertrophy and marked edema (figure 20). The reparative phase consisting of epithelial hyperplasia and marked cellularity of the propria commenced at about the twelfth day (figure 22). The immune phase during which there was restoration of the epithelium and either follicular or mild, focal, diffuse lymphoid infiltration of the propria, started at about the fifteenth to eighteenth day (figure 23). The mucosa appeared to be normal by the twenty-eighth day after vaccination (figure 26).

Of the three groups receiving the combined Newcastle-Bronchitis vaccine, one group (7D) did not exhibit the maximum response until the twelfth to fifteenth day following vaccination, and two groups (6C and 6D) failed to show any response.

The infectivity titer of five of the eight IB vaccines is shown in figure 15. Of the eight vaccines, three were not tested as they were combined Newcastle-Bronchitis vaccines. It is not practical to selectively separate the two viruses to obtain a true quantitative titer of IBV from a combined Newcastle-Bronchitis vaccine. The five vaccines tested had titers that would indicate a virus content sufficient to stimulate immunity in chickens against IBV.

The NI of serum from each group of chicks are presented in figure 16. The positive NI (i.e., 3.0 or over) induced by six of the eight vaccines demonstrates that immunity against IBV was established and confirms the changes observed in the tracheal mucosa. The two vaccines used for groups 6C and 6D produced a NI of less than 1.5 which corresponds to the lack of change in the mucosa for these two groups of chicks.

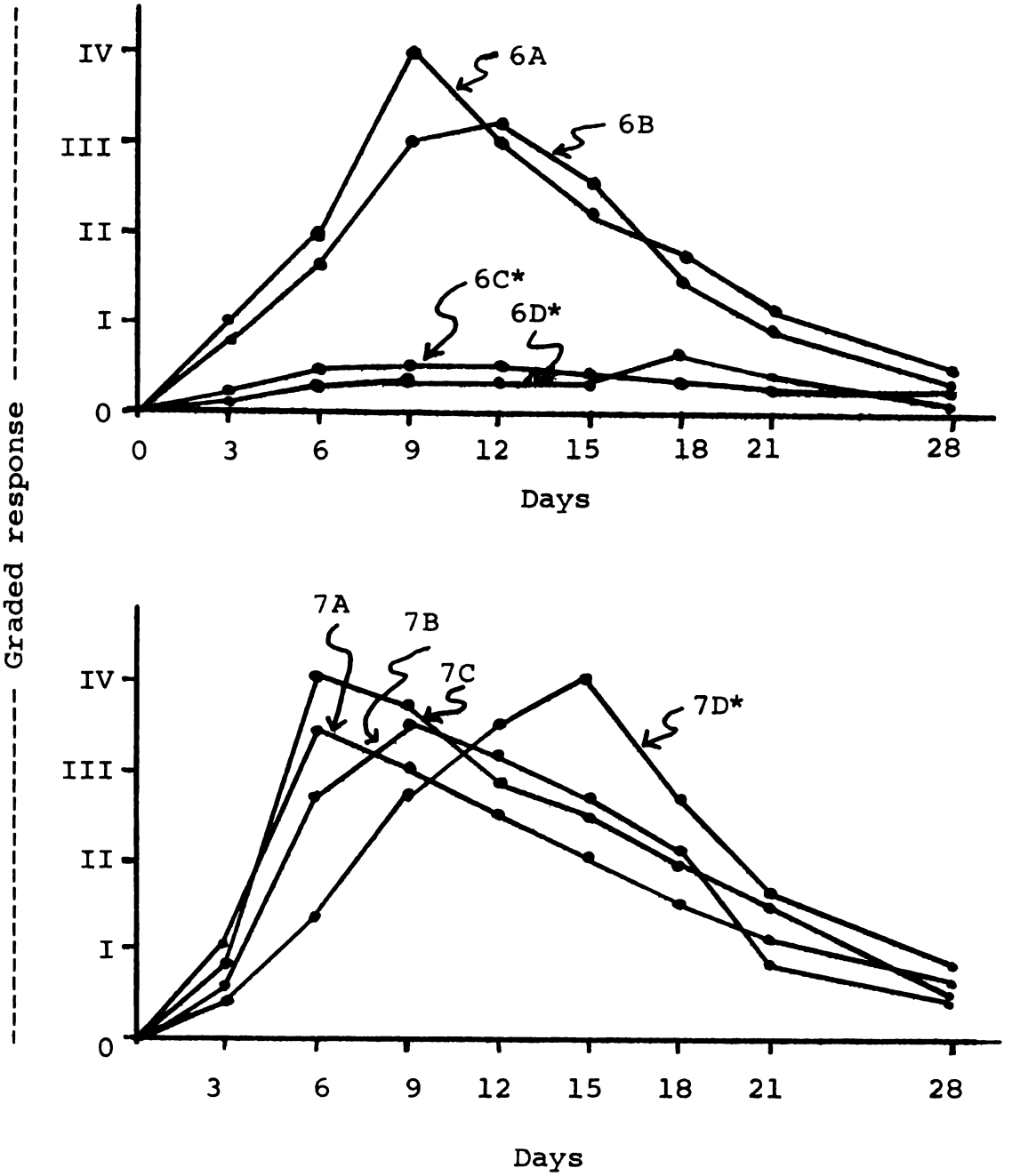


Figure 14. Response of the tracheal mucosa following a single intranasal vaccination of 15-day old chickens with eight serials of IB vaccines.

Legend to grade of response:

0 - No change from normal.
I - Slight edema in mucosa and submucosa
II - Increased edema and congestion of blood vessels.
III - Cellular infiltration and gross thickening of mucosa.
IV - Maximum response noted: much edema, cellular infiltration, and exudate in lumen.

* Indicates combined Newcastle-Bronchitis vaccine.

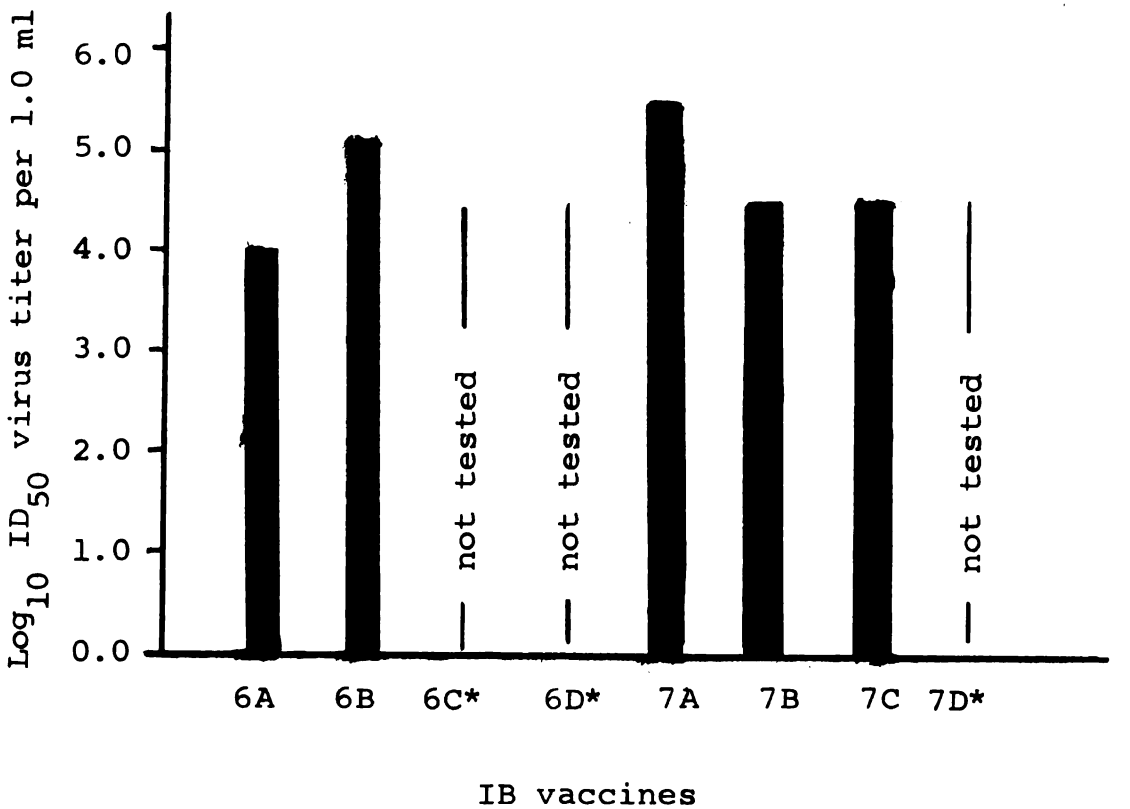


Figure 15. Virus titration results obtained near the date of vaccination of chicken with eight serials of IB vaccines used for evaluation of changes in the tracheal mucosa (figure 14).

* Indicates combined Newcastle-Bronchitis vaccines.

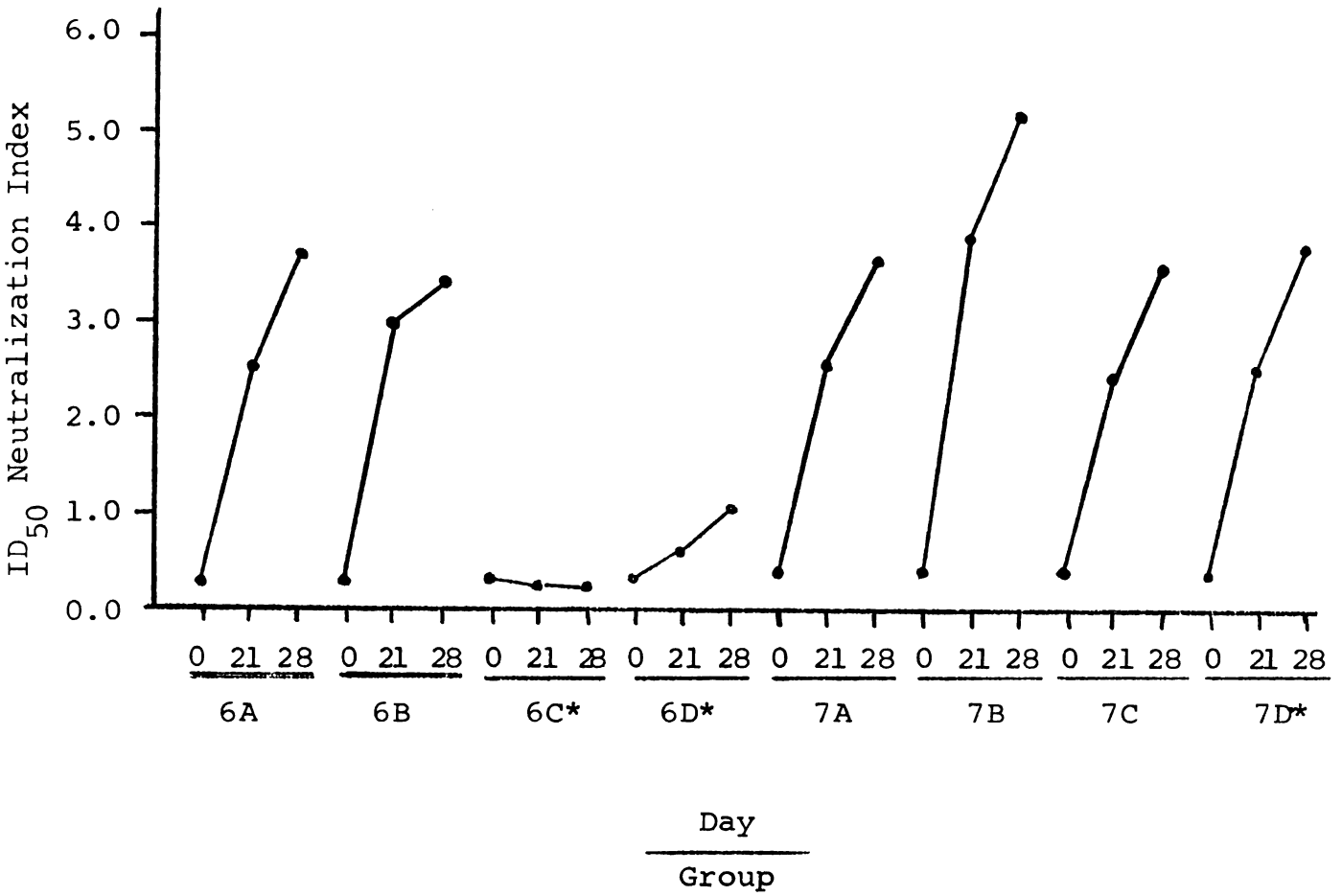


Figure 16. Neutralization indices for serum collected from eight to ten chickens 21 and 28 days after intranasal vaccination of 15-day old chickens with eight different serials of IB vaccines used for evaluation of changes in the tracheal mucosa (figure 14).

* Combined Newcastle-Bronchitis vaccine.

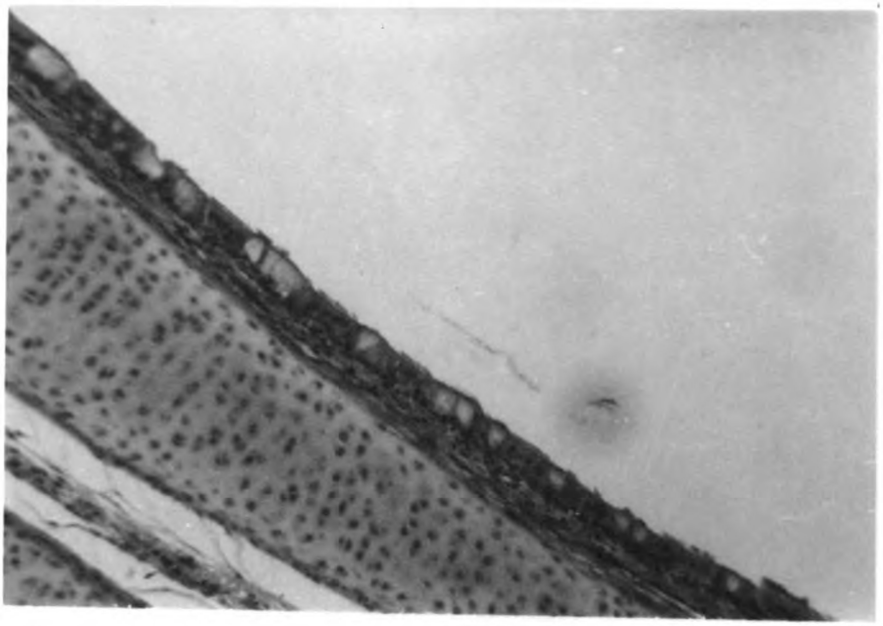


Figure 17. Normal trachea (7E-0) from 15-day old chickens of group 7E at time of vaccination of chickens in groups 7A, 7B, 7C and 7D. x200

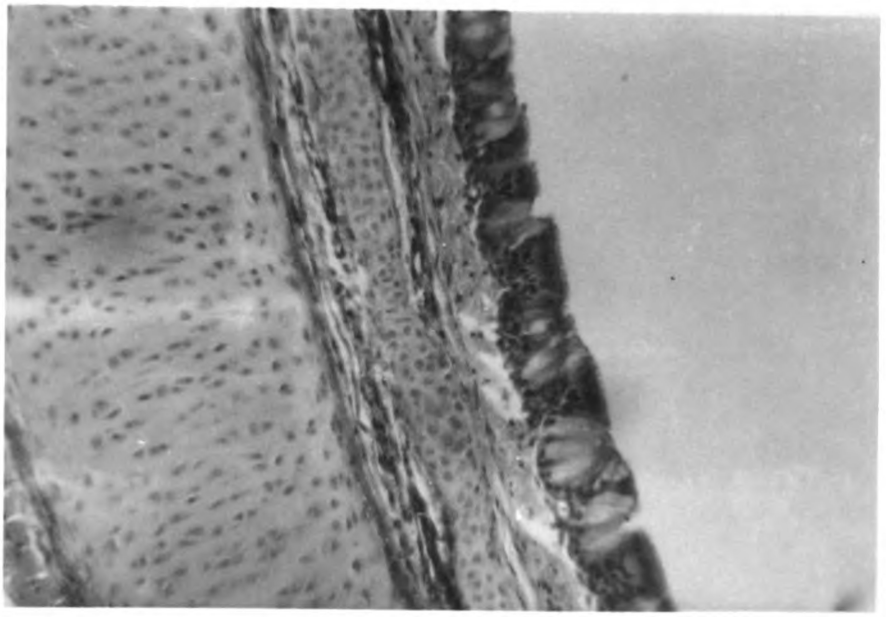


Figure 18. Normal trachea (7E-21) from 36-day old hatchmates of groups 7A, 7B, 7C and 7D 21 days after vaccination of chickens. The mucosa bears pseudostratified ciliated epithelium with goblet cells and mucous crypts. The propria may contain several lymphocytes that may displace some mucosal cells and approach the surface of the mucosa. x200

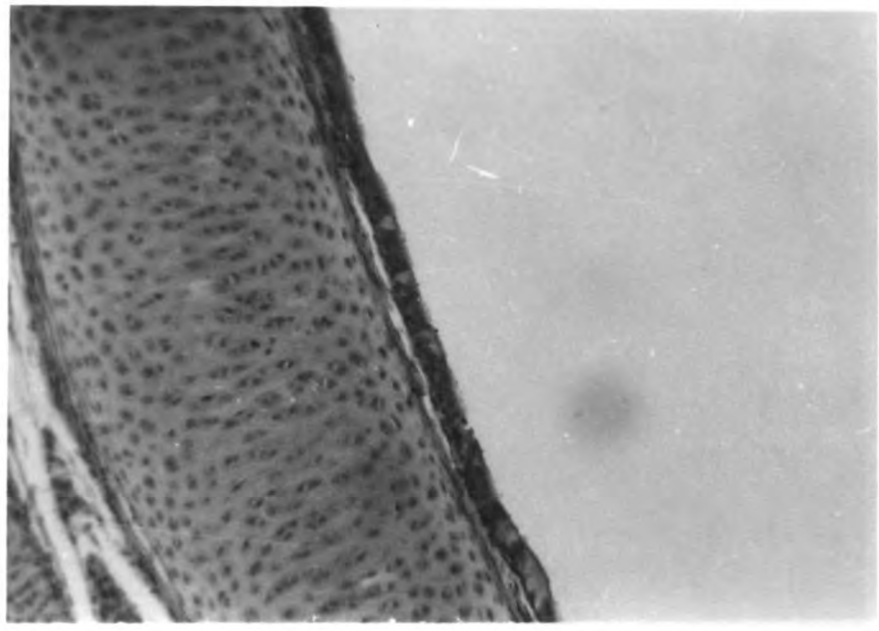


Figure 19. Trachea (7A-3) from chickens of group 7A on the third day following vaccination. Some congestion of the capillaries but not much change from normal.

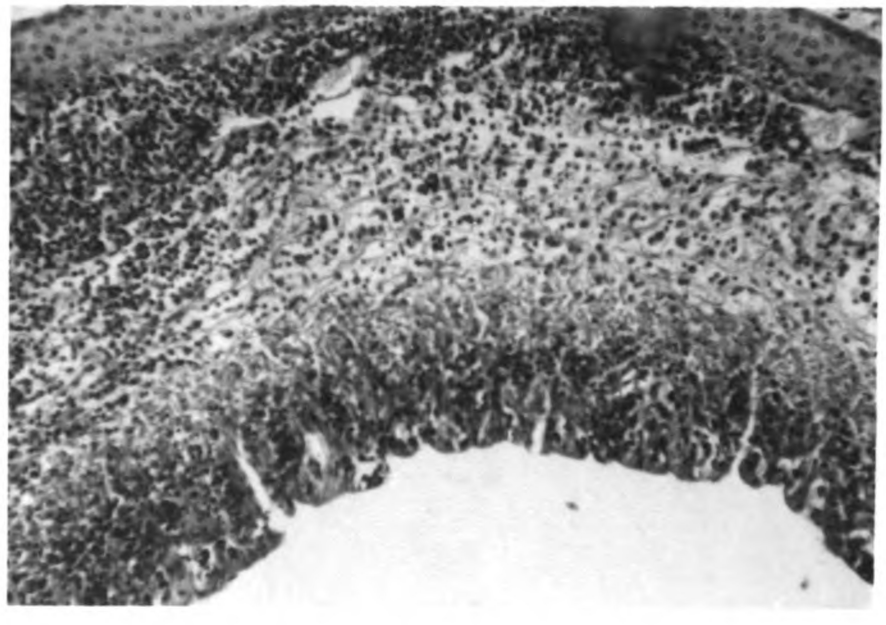


Figure 20. Trachea (7A-6) from chickens of group 7A on the sixth day following vaccination. The massive zone of edema, marked congestion, loss of cilia from epithelium, and thickened mucosa are characteristic of the acute phase. x200

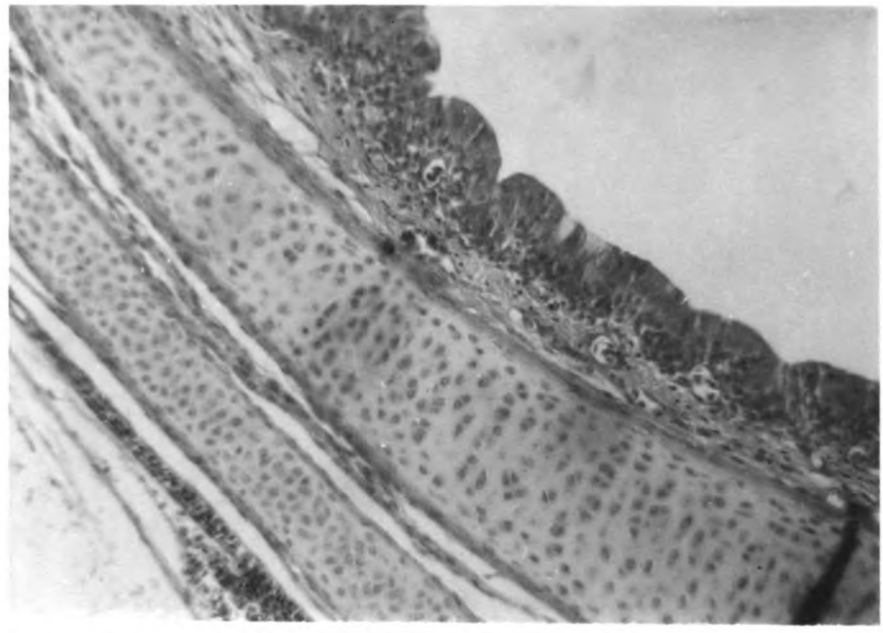


Figure 21. Trachea (7A-9) from chickens of group 7A on the ninth day following vaccination. The decreased depth of edema in mucosa, a cellular infiltration of mononuclear cells, a tendency for intraepithelial glands to form in the mucosa, and a non-cellular exudate in the lumen of the trachea are characteristic of the reparative phase. x200

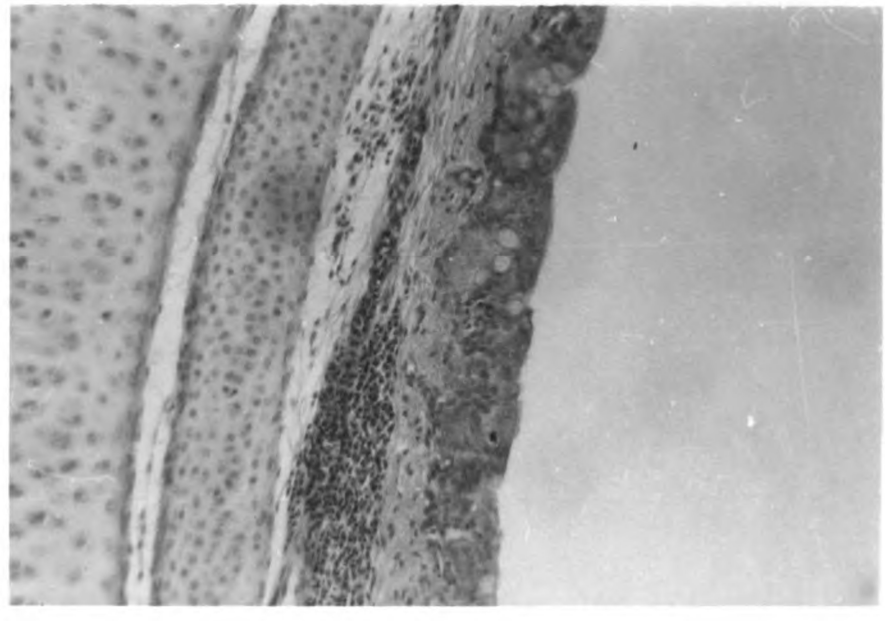


Figure 22. Trachea (7A-12) from chickens of group 7A on the twelfth day following vaccination. An increase in mononuclear cells and repair of the mucosa has started. x200

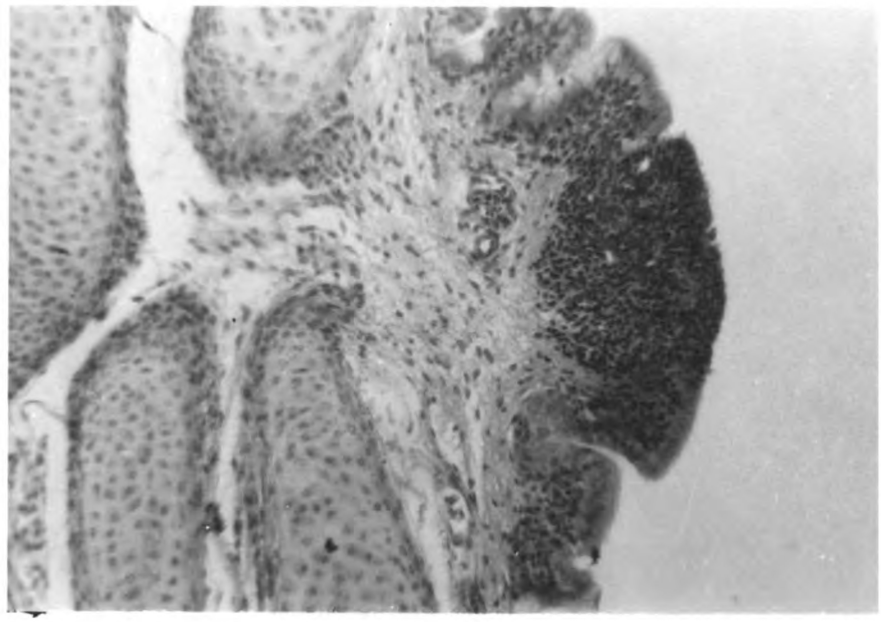


Figure 23. Trachea (7A-15) from chickens of group 7A on the fifteenth day following vaccination. The mucosa has nearly returned to normal thickness but is folded with aggregates of lymphfollicle-like cellular elements accumulated near the surface. This is characteristic of the immune phase. x200

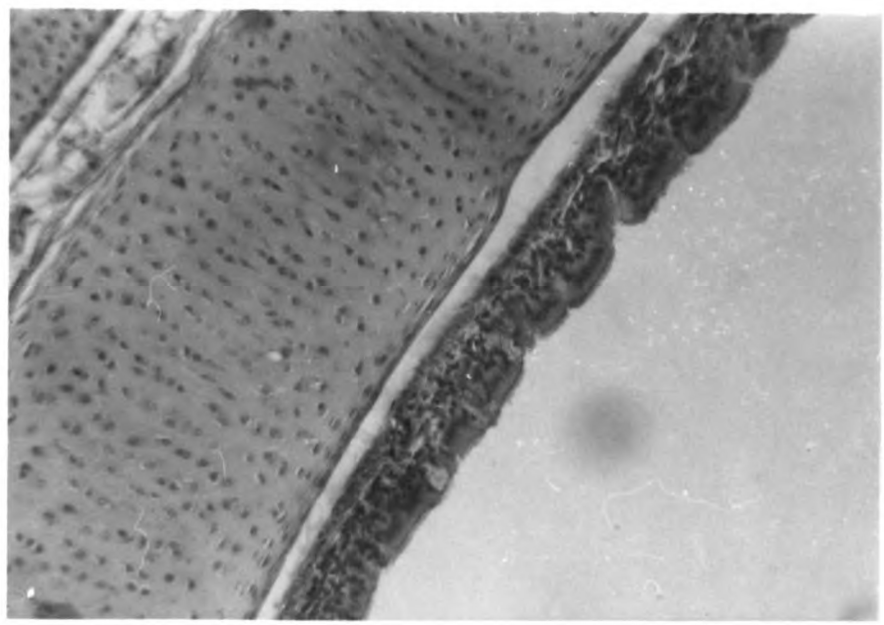


Figure 24. Trachea (7A-18) from chickens of group 7A on the eighteenth day following vaccination. The epithelium has nearly returned to normal with cilia present. x200

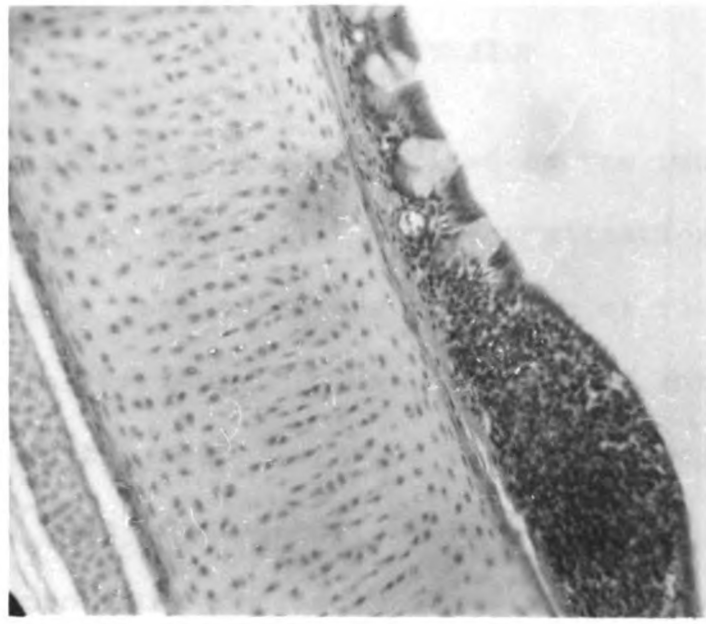


Figure 25. Trachea (7A-21) from chickens of group 7A on the twenty-first day following vaccination. The intraepithelial glands and lymphfollicle-like node present are characteristic of the latter portion of the immune phase. x200

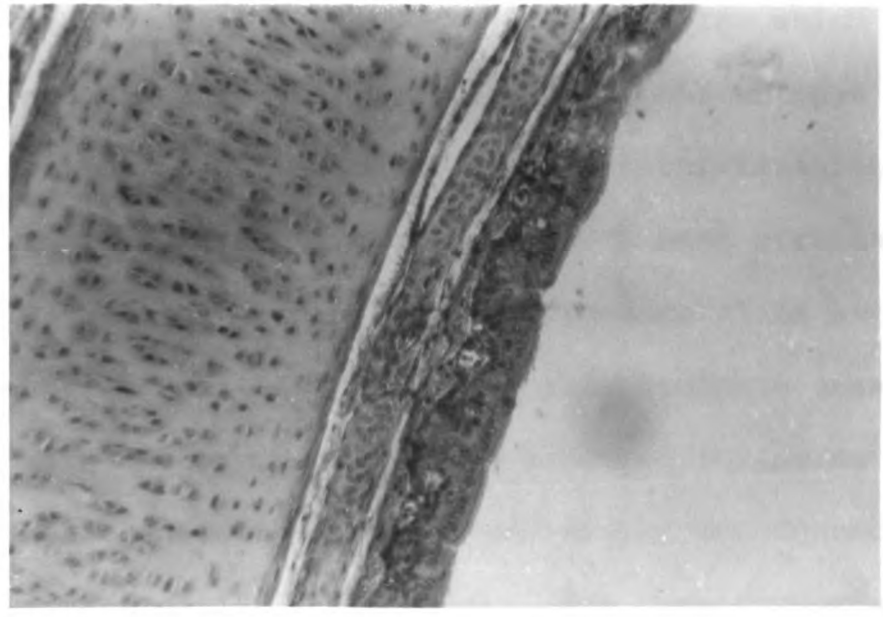


Figure 26. Trachea (7A-28) from chickens of group 7A on the twenty-eighth day following vaccination. The mucosa appears to be nearly normal. x200

DISCUSSION

Neutralization may be defined as the reduction of viral infectivity by antibody. The neutralization index (NI) is a quantitative measure of the reduction of viral activity.

Several variables in methodology and interpretation of results in the neutralization test for assay of immunity against IBV have been considered. These variations indicate that a uniform procedure for assay of antibody against IBV is required if workers in various laboratories are to be able to reach similar conclusions when testing replicate materials.

A. Antigens

The embryo-adapted Beaudette strain of IBV which is non-immunogenic for chickens, can be used as the antigen in the neutralization test to obtain reliable neutralization indices with serum from chickens vaccinated with most strains of IBV currently used in the production of commercial IB vaccines.

However, the Beaudette antigen will indicate negative results with known positive serum produced following vaccination of chickens with vaccines containing the Connecticut (A5968) strain of IBV. Conversely, the Connecticut strain of IBV when used as the antigen in the neutralization test gives negative to weakly positive neutralization indices when used against the heterologous anti-Massachusetts serum and related serotypes.

In the present study the Connecticut antigen tested against anti-Massachusetts serum showed a NI range from 1.7 to 4.8. These results are different than those reported by Jungherr, et al. (1956b) and Hofstad (1958) where the Connecticut antigen used against anti-Massachusetts serum indicated a NI approximately equal to that for normal serum.

The Massachusetts strain of IBV was maintained by serial passage in chickens by using as inoculum a suspension of lung and trachea collected from infected chickens which had been in the Horsfall-Bauer type isolation units. In one instance the chickens were maintained concurrently with other chickens in adjacent units inoculated with a virulent strain of IBV previously isolated at Michigan State University. The lung and trachea were harvested in the morning from chickens inoculated with one strain of IBV and in the afternoon from chickens inoculated with the other strain of IBV. Careful techniques were observed and it is not considered likely that cross-contamination of the strains occurred. It is not known whether the strain isolated at Michigan State University will induce neutralizing antibodies against the Connecticut strain.

At the time that the chickens were inoculated for production of anti-Massachusetts serum used in this study, other chickens in adjacent isolation units were inoculated for production of anti-Connecticut serum with the Connecticut strain

of virus contained in a commercial vaccine. Chickens of both groups were inoculated several times for purposes of hyperimmunization.

Assuming that cross-infection of the chickens with either strain of IBV did not occur since serum from control chickens kept in adjacent isolation units did not demonstrate an antibody level above that for normal serum against either antigen at any time, it would seem that one of the following may have occurred:

(1) repeated inoculation of chickens with the Massachusetts strain may have stimulated production of sufficient antibody to partially neutralize the heterologous Connecticut antigen, or

(2) modification of the immunogenic properties of the Connecticut antigen, which had undergone nine to eleven serial embryo passages by the allantoic cavity route subsequent to its recovery from a vaccine, may have resulted in a higher percentage of "D" phase particles such as those contained in the embryo-adapted Beaudette antigen (Singh, 1960). The heterologous anti-Massachusetts serum may have neutralized the "D" phase particles only contained in the Connecticut strain of virus.

Of the two possibilities, modification of the immunogenic properties of the Connecticut antigen as the result of continued serial embryo passage is considered to be the most likely explanation for the demonstration of a NI above 3.0, which is considered the base line for immunity induced by IBV, when tested against the heterologous anti-Massachusetts serum.

This variation in neutralization appears to be related to the embryo passage level of the Connecticut antigen. With the virus at about the fifth embryo passage following isolation from a vaccine, neutralization by the Massachusetts serotype did not occur. With the virus at the ninth to eleventh embryo passage, neutralization did occur but not to the same quantitative level as with antibody homologous to the virus.

These differences in neutralization of the Beaudette antigen by antiserums produced by serotypes such as the Massachusetts and Connecticut strains emphasize the need for further studies on the antigenic and immunogenic properties of strains of IBV and the specificity of the antigen-antibody reactions (Cunningham, 1960a).

Neutralization indices may be influenced by the titer of the virus-control mixture for the test. A virus with a titer below $10^{6.0}$ per 0.1 ml can limit the possible range of the NI and result in a low NI recorded for a serum. With a virus of higher titer, a higher NI is usually obtained but a virus

titer exceeding $10^{8.0}$ or $10^{9.0}$ per 0.1 ml would appear to permit a NI above the optimum range as a measure of immunity by vaccination with IBV when correlated with vaccination-challenge techniques.

Occasionally, serum-virus titers less than 1.0 are used to calculate the NI. These titers are misleading for purposes of critical assay because a definite end-point calculated by the method of Reed and Muench is not possible.

As experienced in many laboratories, the thermolability of the Beaudette antigen has made the serum-dilution method for the neutralization test in embryos (constant virus-varying serum dilution technique) impractical because the results obtained have been difficult to replicate.

The influence of time and temperature on inactivation of the Beaudette antigen during the neutralization test was investigated. Holding the viral mixtures for the test at room temperature may adversely affect the results of the test unless thermal inactivation of the viral infectivity of the antigen is controlled. A decrease of 0.3 log unit in the virus titer represents a 50% decrease (half-life) in the infectivity of the viral population.

In addition to accuracy of operations with pipettes and syringes, speed when handling the antigen was emphasized as the infectivity of the Beaudette strain as contained in

undiluted allantoic fluids has been reported (Singh, 1960) to decrease $10^{1.3}$ in three hours at 37 C.

When the serial dilution virus-control mixtures at the beginning and at the end of a test were titrated, the decrease in infectivity of the antigen at 4 C was $10^{0.9}$ in 40 minutes when nutrient broth containing antibiotics was used as the diluent.

The minus NI for serums from control chickens appeared to be due to the effect of the rapid inactivation of the virus-control which was inoculated last. Since normal avian serum appeared to stabilize the infectivity of the antigen for a sufficient time for performance of the test, the use of normal horse serum (2% in nutrient broth) was investigated.

When performed at 4 C, there was no inactivation of the antigen in the virus-control mixtures evident during the time required for performance of the test. With one test performed at room temperature (20 C) with normal horse serum in the nutrient broth, the inactivation of the antigen was similar to that when nutrient broth alone was used at 4 C.

Normal horse serum in a final concentration of 2% in nutrient broth containing antibiotics appears to have a definite stabilizing influence on the inactivation of the Beaudette antigen at 4 C and should eliminate the minus NI sometimes reported when normal avian serum is tested.

Further studies with readily available diluents such as tryptose broth, peptone broth, and others with the addition of various concentrations of serums of other species should be made to ascertain the most desirable diluent to be employed for the test.

B. Serology.

The NI of an individual serum may show that a vaccination "take" has occurred. If the entire flock is sampled, the percentage of chickens protected may be determined. The testing of individual chickens representing a small percentage of the flock may give misleading results unless careful random sampling by an approved statistical method is used.

On the basis of the results obtained in this study, an individual serum with a high NI may have sufficient antibodies to obscure the low NI of another serum when the serums are pooled and tested. The mean of the antilogarithms of the NI of individual serums is not always the same as the antilog of the NI of the pooled serums.

In a testing program used to evaluate the efficacy of IB vaccines, 80% of the serum samples are expected to exceed a NI of 3.0 (Manual for the Examination of Poultry Biologics, 1959). Equal quantities of serum from two chickens are often pooled for economy of time and expense and because the amount of serum collected from young chicks may be insufficient for individual testing.

By this procedure, 8 of 10 individual serum samples, or 4 of 5 pooled samples from two chickens, would be expected to exhibit a NI above 3.0 for a vaccine to be considered satisfactory for potency. However, even though 4 of 5 pooled serums may exhibit a NI above 3.0, any one or all of these four satisfactory pools may include a serum with a low NI that was obscured by the high NI of its companion serum and thus falsely indicate satisfactory immunization has been established.

The NI of pooled serums can be used as a reasonably good measure of immunity but would not be as accurate as the results obtained from individual samples.

For evaluation of efficacy, IB vaccines are usually administered to one- to seven-day old chicks and antiserum collected for assay of immunity 21 days following vaccination. The amount of serum collected at this time is often insufficient for testing due to the age and size of the chicks. Bleeding of chickens 28 days after vaccination may be permitted to collect sufficient serum to obtain the minimum NI of 3.0 required of 80% of the chickens tested.

This present study indicates that the NI continues to increase from about 0.5 to 0.9 log units during this additional week. If bleeding at 28 days is to be established as a standard procedure, then the minimum NI should be increased at least 0.5 log units to a minimum NI of 3.5 at 28 days after

bleeding as the criterion of an adequate immunity induced by the vaccine.

The effect of heating the serum sample followed by refrigeration or freezing was examined in the present study.

Several authors have reported that serum used in their studies was subjected to 56 C for 30 minutes while other authors did not indicate if the serum was heated.

Serum components other than antibody can inactivate certain viruses and resemble specific neutralization (Ginsberg, 1960). Complement and properdin have been incriminated in some instances. Another component, the so-called heat-labile inhibitor present in human serum and that of various experimental animals, inactivates influenza, mumps, and Newcastle disease viruses as effectively as do homologous antibodies. These inhibitors can be removed by heating the serum at 56 C for 30 minutes. These substances have caused serious difficulty and can lead to the assignment of etiological significance to a virus when, in fact, no such relationship exists. The effect of these non-specific inhibitors on IBV has not been reported but the loss of 0.5 to 1.5 logs of neutralizing capacity following heat inactivation of serum has been reported (Markham, 1955).

The present study confirms the loss of neutralizing capacity indicated by Markham. From 0.5 to 1.1 log units decrease in the NI of serum subjected to 56 C for 30 minutes

and stored at either 4 C or -27 C can be expected when compared with non-heated serum stored under the same conditions.

A difference of this magnitude is probably unimportant when dealing with homologous serums of high titer, but it is important when serum of low titer is tested for an NI of 3.0 or better to determine the efficacy of an IB vaccine.

Non-heated normal chicken serum may have an NI not to exceed 1.5. This NI indicates that normal serum may reduce the viral activity of the antigen and reinforces the argument that serum to be used in the neutralization test for IBV should be subjected to 56 C for 30 minutes to reduce the possible effect of heat-labile, non-specific inhibitors.

The method of storing antiserum prior to testing has some influence on the NI obtained for that serum. However, serum stored at 4 C for as long as 36 weeks after collection had a NI approximately equal to the duplicate serum sample stored at -27 C.

Freezing the serum is the preferred method of storage if the serum is not expected to be tested within a week or two. Serum stored at 4 C may become contaminated by mold or bacteria. If the serum is not stored in sealed containers, it is subject to evaporation while in the refrigerator.

C. Histopathology

Changes in the tracheal mucosa following intranasal administration of IB vaccines indicate that this procedure may be used for a critical qualitative assay of immunity induced by vaccines.

The histopathological response of the tracheal mucosa between the sixth and ninth day following administration of IB vaccines may be used for qualitative assay of infection with IBV but may not be used as a criterion for quantitative assay of immunity induced by IBV.

The histopathological changes of the mucosa and submucosa during a 21-day period show acute, reparative, and immune phases directly related in infection and antibody response. These changes are maximum between the sixth and ninth day following intranasal administration of an IB vaccine.

The delayed maximum response between the twelfth and fifteenth day exhibited by the tracheal mucosa of group 7D may have been due to either the Newcastle disease virus component of the combined Newcastle-Bronchitis vaccine, virus interference, or some other factor.

The lack of response of the tracheal mucosa of groups 6C and 6D and a low NI equal to that for normal serums emphasizes the positive correlation between the tracheal responses of the other six groups and the corresponding high NI (over 3.0) obtained from serum collected from these six groups.

The vaccines used for groups 6C and 6D were delayed en-route in shipment and it is possible that this may have had some deleterious influence on the vaccine.

SUMMARY

1. Antibodies against the Massachusetts and related serotypes of infectious bronchitis virus may be detected by the Beaudette strain of infectious bronchitis virus used as the antigen for neutralization tests.

2. Antibodies against the Connecticut serotype are not detected by the Beaudette antigen.

3. Neutralization of the Connecticut antigen by anti-Massachusetts serum is apparently related to the embryo-passage level of the antigen. With low embryo-passage virus, neutralization does not occur. With higher embryo-passage virus, neutralization does occur, but not to the same quantitative level as with antibody homologous to the virus.

4. A virus with a titer below $10^{6.0}$ per 1.0 ml can limit the possible range of the neutralization index and result in a low neutralization index recorded for a serum.

5. Serum-virus titers less than 1.0 should not be used for calculation of the neutralization index in critical assays.

6. The neutralization test is not technically difficult to perform but it does require accuracy in all steps of the procedure. The test should be performed as quickly as possible to minimize thermal inactivation of the antigen.

7. Thermolability of the Beaudette antigen emphasizes that for best results from the neutralization test all

ingredients should be kept in an ice bath during the time required for the test.

8. Normal horse serum in a final concentration of 2% in nutrient broth has a definite stabilizing influence on the infectivity of the Beaudette antigen at 4 C and it is recommended as the diluent for the neutralization test to minimize thermal influences.

9. In only about 70% of the tests can the mean of the antilogs of the individual neutralization indices be used with the reasonable certainty to predict the antilog of the neutralization index of the pooled sample.

10. The use of individual serum samples rather than pooled serum from two chickens is more accurate in determining the efficacy of IB vaccines.

11. The neutralization index of serum collected twenty-eight days after vaccination is from 0.5 to 0.9 log unit greater than the neutralization index of serum collected twenty-one days after vaccination.

12. Normal avian serum may contain non-specific inhibitors which may influence the infectivity of the Beaudette antigen in the neutralization test and adversely affect the NI.

13. Serum subjected to 56 C for 30 minutes and stored at either 4 C or -27 C has a neutralization index from 0.5

to 1.1 log units lower than non-heated serum stored under the same conditions.

14. Serum should be subjected to 56 C for 30 minutes before storage and use in the neutralization test to minimize the effect of non-specific inhibitors.

15. Freezing serum for storage has a slight protective value on the neutralization index, but serum stored at 4 C for as long as 36 weeks after collection has a neutralization index approximately equal to a duplicate serum sample stored at -27 C for the same time.

16. Serum which is not to be tested within a week or two after collection, whether sterilized by filtration or not, should be stored at -27 C to minimize evaporation of the serum and growth of contaminating organisms when stored at 4 C.

17. Intranasal administration of an infectious bronchitis vaccine to susceptible 15-day old chickens produces histopathological changes of the mucosa and submucosa showing acute, reparative, and immune phases during a twenty-one day period. These cyclic changes are related directly to infection and antibody response and are maximal between six and nine days after vaccination.

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