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Avian Infectious Bronchitis Virus Infections in chickens: Virus Excretion and Immune Response

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MASTERS OF SCIENCE THESIS

AVIAN INFECTIOUS BRONCHITIS VIRUS INFECTIONS
IN CHICKENS: VIRUS EXCRETION
AND IMMUNE RESPONSE

BY
ILENE DEBBIE ARNOLD

RECEIVED:
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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
IN
ANIMAL PATHOLOGY

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UNIVERSITY OF RHODE ISLAND
UNIVERSITY OF RHODE ISLAND
1984

MASTER OF SCIENCE THESIS

OF

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1984

ABSTRACT

Avian infectious bronchitis virus (AIBV), a coronavirus, causes acute respiratory disease in chickens.

A rapid diagnostic method was developed for the detection of AIBV in infected chickens using a modified indirect double antibody sandwich (MIDAS) enzyme-linked immunosorbent assay (ELISA). The system was compared with isolation of virus by the conventional use of chicken embryos.

The MIDAS ELISA was as sensitive as isolation of virus with the use of chicken embryos. However, the MIDAS ELISA was more rapid and easier to apply than the use of chicken embryos.

In detecting antibody against AIBV, a modified indirect (MI) ELISA was also developed. The MI ELISA was used to study the immune response of experimentally infected chickens. Results indicated that antibody was not detected in chickens prior to infection, however, the antibody increased in titer as the infection progressed. With increased antibody levels in chickens, shedding of the virus in the trachea and the intestines gradually decreased and disappeared, except in two of eleven chickens whereby

virus shedding persisted in the intestines for as long as twenty-eight days and in the presence of humoral antibodies.

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I would like to express my gratitude and my appreciation to Dr. W. Cheng for his assistance and instruction. I would also like to thank Dr. V. J. Yates for his guidance. I would like to thank my friends and colleagues for their much needed help, understanding, and patience through all the long hours and the bad times. Special mention goes to the people who opened their home to me, put up with all my complaining, and made my stay in Hawaii Island a pleasant one. I would also like to mention my Grandmother and my brother Arnold; my three brothers, Glen, Paul, and Alan; my sister, Elaine; and thank them for the love and support they have given me. Most of all, I wish to thank my Mother and Father, for without their love and support, I would not be where I am today.

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PREFACE

All my work and effort is dedicated in loving memory of my Grandmother Fannie and my Grandfather Samuel; two wonderful people who helped to shape my life and character. They instilled in me the all important attitude . . . "never say can't, because you can do whatever you want to do, . . . you can!"

They even left me with a comforting thought which I wish to share, it was a poem written by Clare Harner Lyon:

Do not stand at my grave and weep;
I am not there. I do not sleep;
I am a thousand winds that blow.
I am the diamond glints on snow.
I am the sunlight on ripened grain.
I am the gentle autumn's rain.
When you awaken in the morning's hush,
I am the swift uplifting rush
Of quiet birds in circled flight.
I am the soft stars that shine at night.
Do not stand at my grave and cry:
"I am not there. I did not die."

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In April of 1930, Schalk and Wason (2) were the first to describe and report "an apparently new respiratory disease of baby chicks" in North Dakota and throughout the North Central region of the United States. In 1936, Beaudette and Daniels (3) established that the etiologic agent was a filterable virus. Beaudette and Hudson (4) were the first to cultivate the virus in embryonated chicken eggs. With the establishment of avian infectious bronchitis as a distinct disease, a considerable body of information on the morphological and biological properties followed due to rapidly developing technology and research techniques.

Avian infectious bronchitis (AIB) is an acute, highly contagious, viral respiratory disease of chickens characterized by respiratory distress, tracheal rales, coughing associated with the contraction of various muscles in the abdomen, and sneezing. In young chicks, there may be a

LITERATURE REVIEW

I. Introduction.

Avian infectious bronchitis virus (AIBV) causes a disease which has emerged within the last four decades from the melange of the "respiratory disease complex" of poultry to a well-defined and separate entity (1).

In April of 1930, Schalk and Hawn (2) were the first to describe and report "an apparently new respiratory disease of baby chicks" in North Dakota and throughout the North Central region of the United States. In 1936, Beach and Schalm (3) established that the etiologic agent was a filterable virus. Beaudette and Hudson (4) were the first to cultivate the virus in embryonated chicken eggs. With the establishment of avian infectious bronchitis as a distinct disease, a considerable body of information on the morphological and biological properties followed due to rapidly developing technology and research techniques.

Avian infectious bronchitis (AIB) is an acute, highly contagious, viral respiratory disease of chickens characterized by respiratory distress, tracheal rales, coughing associated with the accumulation of excess mucus in the bronchi, and sneezing. In young chicks, there may be a

nasal discharge, and in laying flocks there may be a precipitous drop in egg production.

Avian infectious bronchitis virus (AIBV) is of great economic importance to the poultry industry. In young chicks there may be high morbidity, and in some instances, mortality, and a debilitating effect which results in decreased weight gain and feed efficiency. In laying flocks the major loss is decreased production and poor quality of eggs due to ovarian damage caused by the disease. Because of its highly transmissible nature and world wide prevalence, AIB is a constant threat to unvaccinated flocks. Immunization programs are costly to the poultry producer. Plus, the stress from natural infection or from vaccination may be a predisposing factor to other infectious outbreaks.

Presently, AIBV is not known to be of public health significance, although "bronchitis-like" agents have been isolated from human respiratory disease such as the common cold (5,6,7) and low neutralizing antibodies titers have been detected in blood from people associated with poultry (8).

Cunningham (1) and Hofstad (7) have published review articles on AIBV.

II. Etiology.

A. Classification.

AIBV is considered to be a species of the genus

Coronavirus of the family Coronaviridae (9, 10, 11). This group of avian, human, and murine viruses have AIBV as the prototype (12). The name "coronavirus" was selected because of the characteristic resemblance of the viruses to the solar corona.

B. Morphology.

The virus particles tend to be generally spherical, but also pleomorphic with characteristic club-shaped projections uniformly distributed on its surface. The size of the particles range from 60-160 nm in diameter (9,12,13, 14). The surface projections are 20 nm long with a 10 nm wide bulbous dilation of the distal end (12). The bouyant density of the virus from allantoic fluid of embryonated eggs ranges from 1.12-1.22 g/ml (9,15) with a major peak at density 1.17-1.18 g/ml (16) in sucrose. The sedimentation constant is 344 S (17).

C. Viral composition.

The nucleic acid of AIBV is ribonucleic acid (RNA). The genome is the largest viral RNA known to date. It is single-stranded, polyadenylated, nonsegmented RNA which is of positive polarity and is therefore infectious (18,19,20). The estimated molecular weight of the genome ranges from about 3×10^6 to 9.0×10^6 (19,20,21).

AIBV synthesizes six discrete species of virus-specific RNA. These comprise the genome and five single-

stranded RNAs ranging in molecular weight from 0.8×10^6 to 2.6×10^6 . All of the smaller RNAs are subgenomic and are probably viral mRNA's with specific functions (20).

The polypeptide composition of AIBV has been reported to contain as few as three and as many as sixteen structural proteins in the virion (16,22,23,24,25).

The susceptibility to ether indicates an essential lipid (14) in the envelope of the virus.

The cellular receptors of AIBV are complex and contain thiol (SH) groups and N-acetylneuraminic acid (NANA) as an integral part of the receptor sites (26). The neuraminidase-like activity is associated with the hemagglutinin of AIBV (27).

D. Antigens.

There are at least three antigenically distinct soluble virus-specific AIBV antigens in viral allantoic fluid. The viral antigens are smaller in size than the virus particle. Antigen 1 is the smallest and seems to be made up entirely of protein. Antigen 3 is the largest and like antigen 1 is distributed over the surface of the virus particle. Antigen 2 is a ribonucleoprotein that resides in the virion (28).

E. Resistance to physical and chemical agents.

Most strains of AIBV in allantoic fluid are inactivated at 56°C in 15 min, but some strains can survive for a longer period of time (29). AIBV stores well at low

temperatures as infected allantoic fluid (1,7) and can be stored indefinitely if lyophilized.

The optimal pH 7.8 gives the virus maximum stability.

F. Replication.

The site of multiplication is in the cytoplasm. AIBV enters the cells of chicken chorioallantoic membrane by viropexis. Virus uptake is triggered by attachment of the virus to the cell membrane (30). Maturation occurs in the cytoplasm and mature virions bud through the endoplasmic reticulum. No budding at the plasma membrane has been observed. Replication of the virus requires a functionally competent cell nucleus and cellular polymerase II (31).

G. Strains.

Prior to 1956 AIBV was considered to be caused by a single antigenic type. However, since that time a number of isolates have been shown to be antigenically different. Using neutralization and chicken immunity tests, the following serotypes of AIBV have resulted: Massachusetts (M-41), Connecticut (M-46), Iowa 97, Iowa 609, Gray, Holt, JMK, RPL, New Hampshire EF, Clark 333, SE 17, Florida, Arkansas 99, Delaware 2868, Delaware 2897, Wachtel, I 1731, Indiana, Maine 209, Holland 52, Italian, Cuxhaven, and Australian "T" strain (17,32,33,34,35,36,37,38,39,40,41) are a few. The Beaudette (M-42) strain is antigenically related to M-41 and it has been used extensively as the

reference virus for neutralization tests. There is still a great need for a systematic approach to the classification of AIBV in order to study the antigenic and the immunological differences among the many subtypes.

H. Laboratory host systems.

AIBV grows in 9-, 10-, and 11-day-old embryonated chicken eggs. The characteristic embryo changes are seen several days postinoculation (PI). During candling the embryo may appear sluggish and weak. Upon opening the egg the embryo is seen "curled" in a spherical ball with a wry neck and deformed feet compressed over the head. A thickened amnion is closely adherent to the embryo. The yolk sac appears shrunken and an increased volume of usually clear allantoic fluid is present. A consistent internal lesion of the bronchitis-infected embryo is the persistence of urates in the mesonephron.

When the virus is injected into the chorioallantoic sac (CAS), the highest concentration of virus is recovered from the chorioallantoic membrane (CAM), followed by the allantoic fluid, amniotic fluid, and liver. Maximum titer of virus was detected in eggs incubated at 37°C for 30 hours (42).

The virus can also be grown in primary chicken embryo kidney cells (43).

I. Interference and Synergism.

Interference with the production of Newcastle

disease (NDV) by AIBV has been demonstrated to occur in chickens, chicken embryos, and chicken kidney cells (1). Avian encephalomyelitis (AE) virus interferes with AIBV in chicken embryos (44). The mechanism of interference has not been clearly defined.

Coexistence, but not synergism has been reported for AIBV and laryngotrachitis (ILT). Synergism exists between AIBV and Mycoplasma gallisepticum and Hemophilus gallinarium in chickens (1).

J. Persistence.

Studies assessing the duration of AIBV infections in chickens produced conflicting results. Fabricant and Levine reported the successful isolation of virus from eggs and from tracheal swabs for forty three days and four weeks respectively (45). Other reports showed that virus could be recovered from hens and eggs at six but not seven weeks after infection (46). Virus was also shown to be recovered from lungs up to 21 days; from anterior nares, trachea, kidney, bursa, spleen, and blood up to 14 days; from the liver and brain up to 5 days; and from the caecal tonsils for 14 weeks (46,47).

AIBV may enter organs of the respiratory system directly from outside the body and be distributed to various organs due to the development of viremia. The same virus once invading the blood stream, can find its way back to the respiratory organs with ease (47). On the other hand, virus

was also demonstrated in the contents of the large and small intestines in great quantity for a relatively long period of time and this fact may be important when discussing persistence of AIBV.

III. Pathogenesis and epizootiology.

The chicken is the only natural host for AIBV. All ages, sexes, and breeds are susceptible.

A. Transmission.

The virus in secretions from the respiratory tract of infected chickens spreads rapidly throughout a flock under natural conditions. Chickens develop respiratory signs within 36 hours or more. Airborne transmission is considered to be the natural route of infection, however, the optimal environmental conditions are not known, although, it has been assumed during epizootics, that the virus spreads between flocks where the farms are close in proximity and in the direction of the prevailing winds.

Contaminated feed, water, clothing, and equipment, as well as, the movement of personnel from flock to flock may serve for indirect transmission of the virus.

B. Carriers and vectors.

Vectors do not appear to be a factor in the transmission of AIBV.

Recurrence of AIBV in certain areas or on the same premise year after year indicates that recovered chickens may serve as "carriers" of the virus as an inapparent

infection or that there is a continued cross infection rather than a true carrier state (1).

C. Incubation period.

The incubation period of infectious bronchitis is 18-36 hours, depending upon the dosage and route of inoculation (7).

D. Clinical features.

a. Chicks.

In chicks less than five weeks old, characteristic respiratory signs are gasping, coughing, and tracheal rales, and a nasal discharge. Excessive lachrymation may be observed and occasionally chicks may have swollen sinuses. The chicks appear depressed and many are seen huddled under the heat source.

The morbidity rate is high, and the mortality rate may be as high as 25% or more (1). Feed consumption and weight gain is markedly reduced. If chicks are infected under two weeks of age permanent damage to the ovary and oviduct may occur.

b. Growing chickens.

In chicks over five weeks of age there are tracheal rales with some gasping and coughing. The disease may go unnoticed if the birds are not carefully observed. Rales are usually not heard unless the chickens are handled or the caretaker listens to the flock at night when the birds are quiet.

c. Adult laying flocks.

In adult laying flocks the signs are the same as for the growing birds. In addition the flock usually experiences a drop in egg production. Flocks affected in the latter part of their laying year usually have a marked drop in egg production and a molt. These flocks require long periods of time to recover production and usually become unprofitable. Pullets in good condition may suffer only a slight drop in production and regain normal production within a few weeks after recovery from respiratory signs.

The reduction in egg production, increase in the number of unsettable hatching eggs, and reduced hatchability of those eggs set is reflected in the small, soft-shelled, malformed, and abnormal quality of the first eggs when a flock starts to return to production (1). Shell irregularities in recovered flocks may persist for an indefinite period of time.

The morbidity rate may be high in the growing stock and adult birds, but, the mortality rate is usually low.

E. Gross lesions.

Commonly encountered gross lesions at necropsy of affected birds reveals a serous, catarrhal, or caseous exudate in the trachea, nasal passages, and sinuses. There is also congestion and edema of the lungs, fibrinous inflammation or cloudiness of the air sac membranes with

possible yellow, caseous exudate. Yellow, caseous plugs may be in the lower trachea and bronchi of chicks that die. Catarrhal inflammation of the nasal passages and sinuses is seldom encountered in chickens over two months of age. Small areas of pneumonia may also be observed around the large bronchi (1,7).

F. Immunity.

a. Active.

Active immunity results from the recovery of chickens from natural or artificial infection with AIBV as soon as the symptoms have subsided. The primary antigenic stimulus is accompanied by a marked, but transient increase of total globulin and gamma globulin (48). It requires about three weeks for chickens to reach a high level of antibody following exposure to AIBV. Flocks that have experienced an outbreak of bronchitis have some degree of immunity, and antibodies can be demonstrated for at least a year. However, the antibodies may decline sufficiently for reinfection to occur, especially with an overwhelming challenge dose of virus or experience with severe natural condition. The plurality of the serotypes complicates clinical interpretation of the immune status, particularly with reinfection, as cross immunity is not always effective for subsequent infections (7).

Local tracheal immunity has an important role in resistance to bronchitis. Chickens recovered from aerosol

infection with a high embryo passage virus may be resistant for relatively short periods of time to subsequent challenge with low embryo passage virus even though the neutralizing antibody level is low or not demonstrable (49). This may have something to do with an IgA-like immunoglobulin (50,51, 52,53,54) found in chickens.

Cell mediated immunity (CMI) is also part of the immunological response of chickens to both primary and secondary vaccination. However, there is no direct correlation shown between CMI responses and antibody titers or between degrees of CMI responses to vaccination and challenge and clinical signs after challenge (55).

b. Passive.

Naturally acquired passive immunity results from maternal antibodies in the yolk of eggs laid by hens recovered from natural or artificial infection. Antibodies are demonstrable in the blood of embryos after about 15 days of incubation (56). The antibody levels in the chick is highest soon after hatching, declines steadily to a negligible level by four weeks, and the chick is then susceptible to the virus (57,58,59). Passive immunity serves to reduce the severity of the disease but not to prevent respiratory infection following exposure to the virus.

IV. Diagnosis.

Diagnosis of AIBV must be based on the isolation of

the virus or by serological tests.

Similarities in the early stages of the infection with other disease states such as NDV, ILT, infectious coryza, chronic fowl cholera, fowl pox, vitamin A deficiency, and chronic respiratory disease (60), make it difficult to make a field diagnosis of AIBV.

After the disease has progressed sufficiently, the neurologic disturbances associated with NDV, the forced expiration associated with ILT, and the swollen face and nasal discharge associated with infectious coryza, allow a presumptive diagnosis to be made on the basis of clinical signs, morbidity, and mortality.

A. Virus isolation.

Isolation of AIBV is accomplished by inoculating 9- to 11-day-old embryonated chicken eggs via the CAS with a suspension of lung, bronchii, and trachea from infected chickens in the acute stage of the disease (1). Penicillin and streptomycin are usually used to control bacterial contamination. Identity of the virus is based on gross pathologic alteration. It is often necessary to make two or three passages of the virus before typical alterations are produced. The allantoic-amniotic fluid (AAF) should be collected after three days PI for further passage. The remaining embryonated eggs should be examined six or seven days PI (61).

B. Serology

The diagnosis of AIBV by serologic methods is based on demonstration of antigen or antibody. Each test has its own advantages and disadvantages.

a. Virus Neutralization.

Virus neutralization (VN) is a test which usually employs either the variable virus-constant serum or the constant virus-variable serum procedure (62).

The first sample of blood is collected during the initial stages of the disease and the second sample is taken in two or three weeks. Both samples of serum are tested for neutralizing antibodies in embryonated eggs.

VN is the most widely used serologic test for identification, screening and classifying field isolates of AIBV (63,64). Although this method is effective, it is laborious to set up; expensive and time consuming.

b. Plaque Reduction.

The reduction of plaques in a primary chicken embryo kidney cell (CEKC) culture for the detection of AIBV-neutralizing antibodies has been reported to be a practical test (65). This test is comparable to classical methods of VN which use the embryonated egg for the assay system.

c. Agar Gel Precipitation.

Agar gel precipitation (AGP) is another serologic test which is particularly useful for the rapid, routine differential identification of viruses (66). The material

necessary for the AGP test is inexpensive. The test can be set up and read more quickly than the VN or VI procedures. The AGP test is well adapted for processing large numbers of serum samples. Precipitins appear sooner than significant levels of serum neutralizing antibodies in the majority of birds. Diagnosis can be made in five to eleven days and the test is most efficient during the period after infection when the VI efficiency is decreasing and VN titers are not yet uniformly positive.

The AGP test does have its disadvantages. The test is subject to errors in reading and interpretation. The bronchitis precipitins are detectable for only a relatively short period of time. Plus, the test is not suitable for individual bird diagnosis, since precipitins are not produced in every susceptible bird exposed to AIBV.

d. Immunofluorescence.

The immunofluorescence (IF) procedure has been employed with considerable success for the identification, differentiation, and study of AIBV in infected cell cultures (67). The use of divalent or polyvalent antisera and either frozen sections or impression smears of the tracheal explants may be advantageous for routine diagnosis of AIBV. Cross fluorescence between serotypes is considered to correspond more closely to cross-protection tests in chickens than to cross-neutralization test by PR in CEKC.

The biggest disadvantage with IF is that AIBV does

not grow in cell culture upon initial isolation but requires primary embryo adaptation.

e. Compliment-fixation.

Direct, indirect, and modified direct compliment-fixation (CF) tests have been applied to AIBV (1,68). The CF test is useful in monitoring chicken flocks because antibodies can be detected early after infection and persist for several weeks. An advantage CF has over VN is that more sera can be tested more rapidly than by VN test. However, the CF antibodies may not have virus-neutralizing capability, since they appear very early after infection.

f. Hemagglutination-inhibition.

Although AIBV does not cause direct hemagglutination (HA) of chicken erythrocytes, HA activity can be induced and the virus can be used in a hemagglutination-inhibition (HI) test (69,70). The HI is a simple, rapid, inexpensive, highly reproducible method of measuring antibodies and individual serum testing is possible. It can also be used for multistrain AIBV testing with specificity. It has been used as a virus-typing tool (71). The HI test is also valuable for monitoring the immune status of chicken flocks and the efficacy of vaccination programs (42). However, the surface of the virus must be modified in order to have HA activity and it has been noted that not all strains of AIBV exhibit this activity and not all strains are modified in the same way.

(69).

g. Enzyme-linked Immunosorbent Assay.

Recently, immunoenzyme methods have been recognized as useful serologic tools for determining epidemiological indices applicable to viral, bacterial, and parasitic infections and for the detection of toxins in foods for human consumption (72). Among the most successful methods are those utilizing "labelled" antibodies and antigens.

Enzyme labelled antibodies have been used for many years for the microscopic localization and identification of antigens in biological tissue sections (73). The first enzyme-immunoassay were described in 1971 independently by Van Weeman and Schuurs and by Engvall and Perlmann (74) who coined the name ELISA (enzyme linked immunosorbent assay). A few years later, the technique was adapted for microplate multiple assays by Voller (75). Since then more than a hundred papers have appeared on the subject attesting to its great promise (76).

The ELISA has been shown to be a quantitative, rapid, reproducible, sensitive, specific, and simple test (77,78,79,80) for detecting antigen or antibody. The test depends on two assumptions: (i) that antigens and antibodies can be attached to a solid-phase support yet retain immunological activity and (ii) that either antigen or antibody can be linked to an enzyme and the complex retain both immunological and enzymatic activity (81).

Experience has shown that these assumptions are true for many antibody-antigen systems.

The ELISA test was evolved due to an interest in developing a means of detecting infectious agents without relying on cultivation. With the ELISA, antigen can be detected and quantified using either a competitive ELISA system or a double antibody sandwich method. The competitive assay has a disadvantage in that each different antigen must be coupled to an enzyme. Due to the differences in antigenic structures, different coupling methods may be required. The noncompetitive method, the double antibody sandwich, for detection of antigen is the most useful because it only requires labelled antibody and the same procedure can be used for labelling all antibodies.

The simplest noncompetitive system is the direct or one-antibody sandwich. One disadvantage of this single antibody approach is that it requires the laboratory to have different labelled antibodies for each antigen to be tested. This difficulty can be overcome by the use of the indirect ELISA which uses an enzyme-labelled anti-immunoglobulin. Since a single molecule of unlabelled antibody is able to react with a number of anti-globulin molecules, there is an increase in the number of enzyme molecules bound per molecule of antigen with a resulting increase in sensitivity. Consequently, the indirect ELISA is also more sensitive than its analogous direct system.

However, there is one inherent disadvantage to the indirect system, namely that reagents prepared in two different animal species must be used to prevent the non-specific binding of the anti-immunoglobulin to the solid phase (82).

There is also a great deal of interest in measuring antibody levels, especially, in infectious diseases. The indirect ELISA is the method of choice for the same reasons as expressed above.

As with any type of immunoassay, the theoretical basis of the ELISA test is simple, but much work is needed to determine optimum specifications for a successful routine diagnostic test. In ELISA systems there are countless possible permutations of variable such as the type of solid phase, the characteristics of the antigen/antibody coating on the solid phase, the test conditions, the type of enzyme and its conjugation to antibody or antigen, and properties of the enzyme substrate.

There are some inherent dangers that the ELISA assay system may not be adequately standardized and controlled, with consequent misleading results. All test, therefore, must be related to positive and negative reference samples (81). Also, the methods for interpreting results of the ELISA are without definition and are, completely arbitrary (72). The elimination of operators subjectivity in evaluating results and rationalization of

the assay design are obvious advantages which favor the ELISA, however, the inappropriate expression of data limits the usefulness of the ELISA (83). There should also be some standardization of the procedure so that between-run, between-laboratory, and between-method variations are slight.

V. Treatment.

There is no specific treatment for infectious bronchitis virus. In flocks of young chicks it is helpful to increase the temperature of the room as well as the brooder. Overcrowding should be avoided and drafts should be eliminated. Feed consumption in the flock should be encouraged to avoid excessive loss in weight in the birds. Recovery takes place as the birds acquire an immunity to the virus.

VI. Prevention and Control.

A. Management.

Strict isolation of the flock is the best prevention, along with sound management practices such as adding only day-old chicks as replacement stock and rearing them in isolation. However even with sound management practices, AIB may occur. This has brought on the necessity of using immunization programs in order to control the disease.

B. Immunization.

The immunization against AIBV has been reviewed by

Hofstad (84).

The first immunization procedure used was started in 1941 in the New England states. It consisted of inoculating a small portion of the birds in a flock with a field isolate and allowing natural spread to the rest of the flock. This type of vaccination procedure using pathogenic field strains has been replaced with modified live virus vaccines.

VII. Conclusion

Although there is presently a wealth of information about AIBV, it is evident that much additional information is needed.

Further investigation is required in the area of pathogenesis and epizootiology, the genetics, the chemical composition, as well as the physical properties and biological events of the virus-host cell relationship.

The prime priority of investigators should be to establish the means by which new AIBV isolates can be recognized and categorized as to their serotype and their relationship to one another.

MATERIALS AND METHODS

Virus. The Massachusetts 41 (M-41) strain of avian infectious bronchitis virus (AIBV), a coronavirus, was used throughout this study. The virus was obtained from Cecila Gulka, Department of Animal Pathology, University of Rhode Island.

The virus was propagated in 9-to 11-day-old embryonated, specific-pathogen-free (SPF) (SPAFAS Inc., Norwich, Connecticut) or commercially obtained eggs. The procedure for virus propagation, as described by Rovozzo and Burke's (62), was as follows: 0.1 ml of the virus suspension containing $10^{3.5}$ ELD₅₀ was inoculated into the chorioallantoic sac (CAS) of each egg. Following inoculation, the eggs were incubated at 37°C and candled daily. Embryos found dead after 24 hours postinoculation (PI) were discarded as nonspecific deaths. After 36-48 hours PI, the eggs were chilled to 4°C before the allanto-amniotic fluids (AAF) were collected. The harvested fluid was pooled and clarified at 7,600 x g for 10 minutes (min) in a Sorvall RC-2B refrigerated centrifuge. Aliquots of the virus suspension were then placed in stoppered tubes and kept at -20°C for later use.

The virus was titered in embryonated eggs by the method described by Rovozzo and Burke (62). The end point titer was determined by embryo lethal dose fifty percent (ELD₅₀) and four eggs were used per dilution of virus. For a detailed description of the method, see Appendix A.

Virus Concentration, Purification, and Isolation. Stock virus in AAF was concentrated on a 60% (w/v) sucrose cushion. Six ml of the 60% sucrose was carefully layered in a polyallomer centrifuge tube beneath 28 ml of the virus suspension. The virus suspension was centrifuged in a Sorvall OTD-50 ultracentrifuge using an AH-627 swinging bucket rotor for 1 1/2 hours at 83,100 x g.

The visible band above the cushion was then collected and stored at -20°C. The virus suspension was further purified on a 20, 30, 50, and 60% (w/v) discontinuous sucrose density-gradient. One ml of virus suspension was layered on the top of the discontinuous gradient in cellulose centrifuge tubes. An AH-650 swinging bucket rotor was used in the OTD-50 ultracentrifuge and the virus was centrifuged for 3 1/2 hours at 153,000 x g.

Using a Auto Densi-Flow (Haake Buchler Instruments, Inc., Saddlebrook, N. J.), ten fractions were collected from each centrifuge tube from different centrifugation runs. All fractions collected were checked for their density using a Bausch & Lomb ABBE-3L refractometer. These fractions were then used to check optimal centrifugation

times and to determine the specific density of the virus.

In the determination of specific density of the virus, each of the ten fractions were serially diluted and the ELD₅₀ was determine. The density and their corresponding infectivity titers were then plotted.

In subsequent centrifugation runs, the visible bands were collected, their densities determined, before being stored at -20°C.

Virus neutralization. The Beta procedure using a variable amount of serum and a constant amount of virus was employed. The chicken serum was diluted in two-fold serial concentrations with phosphate buffered saline (PBS) solution. A suspension containing 100 ELD₅₀ of the virus in 0.1 ml was prepared. Using aseptic technique, the virus suspension was added to the serum dilutions in equal amounts. The mixture was then incubated at 37°C for 1/2 hour. For each dilution, 0.2 ml of serum-virus mixture was inoculated into 4, 10-day-old embryonated egg. The eggs were observed daily and embryos that died after 24 hours PI were discarded. All other eggs were incubated for 6 days when they were opened and checked for signs of infection.

Antisera production. Rabbit anti-AIBV was prepared in a New Zealand white rabbit. A blood sample was taken from the rabbit prior to inoculation. Then 1.0 ml of a virus

suspension containing 10^5 ELD /ml (density of 1.17 g/ml) was mixed thoroughly with 1.0 ml of Freund's complete adjuvant until an emulsion was formed. The rabbit was inoculated intramuscularly with 0.5 ml of the virus-adjuvant mixture into each hind leg. Following a two week period, the rabbit was inoculated intravenously in the right marginal ear vein with 1.0 ml of purified virus containing 10^5 virus particles. After another two week period, a small amount of blood was taken for testing purposes, and the rabbit was reinoculated intramuscularly with 0.5 ml of the virus in each hind leg. The rabbit was bled from alternating ears on day 7, 16, 25, 34, 42, and 50 after the final inoculation. The serum was collected.

The blood taken was allowed to clot at room temperature. The tube was then rimmed with a wooden applicator stick and placed at 4°C overnight. The following day, the tube was centrifuged at $400 \times g$ for 10 min. The serum was then carefully collected from the top of the tube, labelled, and stored at -20°C .

The final serum sample was then tested by virus neutralization in eggs. The final serum preparation had a neutralization index of 640. The same serum sample was also tested in a modified indirect enzyme-linked immunosorbent assay (ELISA). The ELISA titer was 131,072.

Immunoelectron microscopy. Electron microscopy was used to

verify the presence of virus at various stages in the study. Three hundred-mesh copper grids (Ted Pella Co.) were cleaned in acetone overnight and then allowed to air dry. Some grids were then coated with a formvar or a nitrocellulose film. Equal amounts of virus suspension and antisera were mixed together and incubated for 30 min at 37°C to allow aggregates to form. Three different methods for negative staining were used. In the first method, 0.025 ml of the virus-antibody mixture was dropped onto a sheet of parafilm. A coated grid was then placed on top of the drop for five minutes with the coated side facing down. The grid was then washed three times in distilled water before staining. A 2% phosphotungstic acid (PTA) solution at pH 7.1 was used to stain the grids for 15 seconds. In the second method, the coated grid was placed on a block of 2% Noble agar and a drop of the virus-antibody mixture was placed on top of the grid and allowed to absorb through the coating into the agar. The grid was then stained as above. In the final procedure, a pseudoreplication method was used. The virus was placed on a block of 2% Noble agar and allowed to diffuse for 20 min. Then a drop of nitrocellulose was placed on top of the block and allowed to air dry for 20 min. The film was then floated off at a 45 degree angle into a dish of PTA. After 1 min, clean grids were placed onto the film and the film was lifted out of the stain by using a small test tube. The stained grids were then viewed

using a Hitachi HS-9 electron microscope operating at an accelerating voltage of 75 Kvolts. (See Appendix B).

Chickens. Two groups of White Leghorn chickens were used. In the early study, chickens raised from commercial eggs were used. In the latter study, chickens raised from SPF eggs were used. All chickens used were hatched and raised in isolation.

Experimental Plans:

Experiment no. 1. When the commercial chicks were one week of age, they were banded and identified with wing bands. At two weeks of age; blood was taken from the brachial veins, a throat swab was taken from the trachea, and a fecal swab was taken from the cloaca of each chick. After three weeks, the chickens in the infected group were inoculated intratracheally with 0.1 ml of AIBV containing 10^4 ELD₅₀ of virus. At 1, 3, 5, 7, 14, 21, and 28 days PI, blood for serum samples, throat swabs, and fecal swabs were taken from five chickens in each group. At 1, 2, 3, and 4 weeks PI two chickens were bled by heart puncture. The chickens were then sacrificed and their necks were broken, and their lungs were removed aseptically.

Experiment no. 2 This experiment was similar to the first one with some slight modifications. SPF chicks were used,

and at 1, 2, 3, and 4 weeks PI the chickens were bled. At the end of week 4, the chickens were reinoculated (RI) intraocularly with 10^4 ELD of AIBV M-41. Then at 1, 7, and 14 days RI, throat and fecal swabs were taken again and at 1 and 2 weeks PI, the chickens were bled for serum samples.

Sample collection. Sterile swabs were used to collect throat and fecal swabs. Hank's balanced salts solution (HBSS) containing 1% fetal bovine serum, and 0.5% PSF (10,000 U/ml penicillin, 10,000 mcg/ml streptomycin, and 100 mcg/ml fungizone) was prepared. In the first experiment, 3.0 ml of HBSS was placed in sterile glass tubes. The swabs were wetted with HBSS and inserted into the trachea or the cloaca of the chicken. The swabs were swirled in the HBSS in the tubes. The swabs were then removed with forceps and the excess moisture removed by pressing the swab against the side of the tube. The tubes were then centrifuged at $190 \times g$ for 10 min. The samples were labelled and stored at -20°C .

The second experiment was similar to the first, except 1.5 ml of HBSS was used and the tubes were centrifuged at $180 \times g$ for 10 min.

The lung tissue that was taken from the chickens were washed once in PBS and then placed in a small beaker of PBS. The tissue was minced with a pair of sterile scissors.

The fluid was collected in a tube and centrifuged at 260 x g for 10 min to remove any red blood cells. The supernatant was collected in a sterile tube, labelled, and stored at -20°C for future use.

The blood collected was prepared for sera the same way as described in the antisera production section, except that it was centrifuged at 260 x g for 15 min, due to the smaller amount of blood collected and the use of small tubes.

Virus isolation. Samples of tracheal and cloacal swabs from the control and infected group were thawed at room temperature and treated at the ratio 1:4 with the PSF to control contamination. One-tenth of a ml of the sample was then inoculated into each of four 10-day-old embryonated commercially obtained eggs. The eggs were candled daily and embryos that died after 24 hours PI were discarded and considered to be nonspecific deaths. After 36 to 48 hours, one of the eggs in the set was refrigerated at 4°C overnight and harvested for used in a blind passage. After the sixth day PI, the remaining eggs were placed at 4°C overnight. The following day, the eggs were opened and examined for any signs of infection such as dwarfing of the embryo. If no infection was apparent, two additional blind passages were made in a similar fashion as described above.

Protein Determination. A micro protein determination kit (Sigma Chemical, Co., St. Louis, MO.) was utilized in order to determine the protein (mcg/ml) of various virus and antibody samples. This assay system is dependant on the tryptophan and tyrosine content of proteins. A Baush and Lomb Spectronic 21 spectrophotometer transmitting at 725 nm was used. The test sample were diluted with a 0.85% sodium chloride solution. To a tube labeled "blank" 0.4 ml of 0.85% sodium chloride solution was added. To the tubes labelled "test" 0.4 ml of the diluted test sample was added. To all tubes 4.4 ml of biuret reagent was added and the tubes were mixed well and allow to stand at room temperature for ten minutes. Next, 0.2 ml of Folin and Ciocalteu's Phenol reagent was added and mixed well immediately after addition. The tubes were allowed to stand at room temperature for thirty minutes. The contents of the tubes were transfered to cuvettes and the absorbance was read at the same wavelength and on the same instrument used to prepare a calibration curve using the blank as reference.

The calibration curve was prepared using a protein standard diluted with 0.85% sodium chloride solution. The absorbance values versus protein concentration was then plotted and the data was extrapolated in order to determine the amount of protein in the test sample used as a standard (See Appendix C).

ELISA reagents.

Antigen preparation. AIBV was inoculated into SPF embryonated eggs. After 36 hours, the eggs were chilled at 4°C overnight. The AAF was then harvested, pooled, and clarified in a RC-2B centrifuge at 7,650 x g for 10 min. The supernatant was harvested and centrifuged for 1 1/2 hours at 76,600 x g in an ultracentrifuge. The pellet was harvested and resuspended in PBS. A protein determination was done and various dilutions of the virus suspension were tested in an ELISA test in order to optimize the antigen sensitivity.

Antisera. Chicken anti-AIBV was obtained from a commercial source (SPAFAS Inc., Norwich, CN.). This antiserum was used in the modified indirect double antibody sandwich (MIDAS) ELISA as the primary antibody. It was also used in the modified indirect (MI) ELISA as a positive control.

The rabbit anti-AIBV serum that was prepared was used in the MIDAS ELISA as the secondary antibody.

Conjugate. Rabbit anti-chicken IgG (Heavy & Light chains) and goat anti-rabbit IgG (Heavy & Light chains) conjugated to horseradish peroxidase (HRP) were obtained from Cappel Laboratories, Inc. (Cochranville, PA.).

Substrate. The substrate-indicator solution was made by

dissolving 20 mg of orthophenylenediamine (OPDA) in 50 ml of phosphate-citrate buffer (pH 5). (See Appendix D). Immediately before using the substrate solution, 0.020 ml of a 30% hydrogen peroxide solution was added at 25°C.

Stop solution. The stopping solution used to stop the color reaction in the test was a two molar solution of sulfuric acid.

Controls. The substrate control consists of substrate-indicator solution and stopping solution.

The conjugate control consists of conjugated antisera, substrate-indicator solution, and stopping solution.

The antibody control in the MIDAS ELISA consists of the rabbit antiserum, conjugate, substrate, and stopping solution.

The positive control antibody in the MI ELISA consists of SPAFAS anti-AIBV. The negative control antibody consists of SPAFAS negative serum or serum taken from one-day old SPF chicks.

The positive control antigen in the MIDAS ELISA consists of concentrated and three times purified AIBV. The negative control antigen consists of AAF prepared in the same manner as the control antigen.

ELISA procedures.

Modified indirect double antibody sandwich method for antigen detection.

The ELISA assay was performed in 96 flat bottom well, polystyrene Immulon 1 microtiter ELISA plates (Dynatech Laboratories, South Windham, ME.). The procedure is as follows:

1. All wells were coated with 0.05 ml of a 1:1,000 dilution of chicken anti-AIBV in 0.1 M carbonate-bicarbonate buffer, pH 9.6 (Appendix D). The plates were covered with aluminum foil and incubated for 1 1/2 - 2 hours at 37°C. After incubation, the plates were washed three times (3X) with PBS-Tween 20 (0.08% [v/v]) using a Dynatech Miniwasher.

2. Antigen dilutions were made in Costar 96 well U-shaped microtiter plates (Bellco Glass, Inc., Vineland, N. J.). Fifty microliters of the antigen dilutions was added to each appropriate well.

3. The first 9 wells of the horizontal row was used for the antigen negative control. The second 9 wells of the row was used for the known positive control antigen. The next rows were used for the negative control throat swab and negative control fecal swab followed by the test throat and fecal swabs and/or a negative control lung and a test lung sample. Of the 3 remaining horizontal wells, one vertical row was used for an antibody control, one for a

conjugate control, and the final row for a substrate control.

4. The plates were covered and the antigen was incubated for 1 1/2 to 2 hours at 37°C. After incubation, the plates were washed 3X with PBS-T.

5. Rabbit anti-AIBV M41 was diluted 1:2,000 with PBS-T. Fifty microliters was added to all the wells except the conjugated and substrate well which received PBS-T. The plates were covered and incubated for 1/2 hour at 37°C. After incubation, the plates were washed 3X with PBS-T.

6. Fifty microliters of a 1:2,000 dilution of the goat anti-rabbit IgG was then added to all the wells except the substrate well which received only PBS-T. The plates were covered and incubated for 1/2 hour at 37°C. After incubation, the plates were washed 3X with PBS-T, followed by a final rinse with PBS.

7. Hydrogen peroxide was added to the substrate indicator solution (see Appendix D) in subdued light. Fifty microliters was added to all the wells on the plate. After a predetermined reference point was reached, the reaction was stopped, and all the samples were read on a Dynatech MicroELISA Minireader MR 590 at 492 nm. The results were recorded (see Appendix E).

Modified indirect method for antibody detection. The procedure is as follows:

1. All the wells were coated with 0.05 ml of a 1:100 dilution of AIBV antigen in 0.1 M carbonate-bicarbonate buffer, pH 9.6. The plates were covered with plastic sealing tape to make a tight seal. The plates were then refrigerated, at 4°C overnight.

2. The plates were allowed to warm up to room temperature. Then they were washed three times (3X) with PBS-Tween 20 (0.08% [v/v]) using a Dynatech Miniwasher.

3. Two-fold dilution of the serum samples were made in Costar 96 U-bottom well microtiter plates using PBS-Tween 20 (PBS-T) as the diluent. Fifty microliters of each dilutions was placed in its perspective well.

4. The plate was set up as follows. The first 9 wells in a horizontal row was used for a known positive control. The second 9 wells was used for a known negative (SPAFAS) control serum. The next 6 horizontal rows contained the samples to be tested. The three remaining wells in the horizontal rows were used for conjugate and substrate controls.

5. The plates were then covered with aluminum foil and incubated for one hours at 37°C. After incubation, the plates were washed 3X with PBS-T.

6. Peroxidase conjugated rabbit anti-chicken IgG was diluted 1:2,000 with PBS-T. Fifty microliters was then added to each well except the substrate control wells. The plates were covered and incubated for 1/2 hour at 37°C.

After incubation, the plates were washed 3X with PBS-T, followed by a final wash with PBS.

7. Hydrogen peroxide was added to the substrate-indicator solution in subdued light. Fifty microliters was added to all the wells on the plate. After a predetermined reference point, the reaction was stopped. The plates were then read on the minireader. The results were recorded (see Appendix F).

Optimization and standardization. All reagents used in the ELISAs were tested for optimal dilution factors and optimal incubation times in order to standardize the tests.

Data interpretation. All positive samples were compared with the negative samples after taking the substrate, conjugate, and antibody absorbance values into consideration. The absorbance values usually range from 0.00-1.99 and a difference of 0.20 between the negative and positive samples was considered significant.

Reproducibility of the results. Test samples were run in duplicate and certain samples were run over a period of time in order to determine the reproducibility of the ELISAs.

Statistical analysis. A one-tailed Students' t test was used to analyze the data (see Appendix G).

RESULTS

Virus concentration, purification, and isolation. The virus suspension was layered on a discontinuous sucrose density-gradient and centrifuged at 153,000 x g for 3 1/2 hours. Ten fractions ranging in densities of 1.07 to 1.27 g/ml were collected. Peak infectivity ($10^{5.5}$ ELD₅₀) correlated with fraction five, which contained a visible band at a density of 1.17 g/ml. Virus was also detectable at densities between 1.13 and 1.23 g/ml. (Fig 1.)

Standardization of the modified indirect double antibody sandwich (MIDAS) ELISA.

Standard MIDAS ELISA. No other investigators have reported on the use of MIDAS ELISA to detect AIBV. Therefore, conditions used for a standard MIDAS ELISA were determined on the basis of its similarity to the modified indirect ELISA. The primary antibody and antigen were both incubated for two hours at 37°C. The secondary antibody and conjugate were both incubated at 37°C for thirty minutes.

Standardization of the primary antibody concentration.

Various dilutions of a chicken anti-AIBV were used to coat the microplates. Due to a low non-specific binding and

high specificity, a chicken anti-AIBV dilution of 1:1,000 containing 51 mcg/ml of protein was chosen. (Fig 2.) This dilution of antibody had a modified indirect ELISA titer of 1/4096.

Standardization of the secondary antibody concentration.

Various dilutions of a rabbit anti-AIBV were used in the standard MIDAS ELISA with a chicken antibody dilution of 1:1,000. (Fig 3.) Due to the availability of a large amount of antibody, a dilution of 1:2,000 was chosen. This dilution contained 124 mcg/ml of protein and had an modified indirect ELISA titer of 1/131,072.

Standardization of the conjugate concentration.

Various dilutions of goat anti-rabbit IgG conjugated with HRP was used in the standard MIDAS ELISA. A dilution of 1:2,000 containing 61 mcg/ml of protein was chosen due to a minimal amount of non-specific binding. (Fig 4.)

Standardization of antibody binding time.

A 1:1,000 dilution of chicken anti-AIBV was used to coat several microplates which were then incubated at various time intervals. A positive and negative reference antigen were then added to the plates and the standard MIDAS ELISA plan was followed. One and one-half hours was chosen due to non-specific binding and sufficient sensitivity. (Fig 5.)

Soula and Moreau (85) demonstrated that the optimal binding time of antibody to antigen occurred in a minimum of thirty minutes, this time was chosen and appeared suitable

for the incubation of the rabbit anti-AIBV.

Standardization of AIBV antigen binding time. Positive and negative reference antigen was incubated at various times in the standard MIDAS ELISA. An incubation time of one and one half hours was chosen due to low non-specific binding and sensitivity in detection of antigen. (Fig 6.)

Effect of time on the conjugate concentration. Using the standard MIDAS ELISA, the effect of time on the sensitivity of the conjugate at a dilution of 1:2,000 was tested. Fig 7. shows an increase in sensitivity as time progresses. An incubation time of thirty minutes was sensitive, had low non-specific binding, and was chosen in the interest of time.

Substrate incubation time. Since it is difficult to achieve constant times and temperatures in all the conditions of the MIDAS ELISA, the substrate reaction time was controlled by the inclusion of a reference antigen on each plate. When the reading of the reference sample reached a predetermined point, the reaction was stopped.

Reproducibility of the results. A positive reference antigen (10⁴ ELD) was retested over a period of a month. Comparable results with no significant variability were obtained.

Standardization of the modified indirect (MI) ELISA. The MI ELISA for AIBV antibodies has been performed by several

investigators (68,78,79,86,87,88,89,90,91). Optimal antigen binding time was reported to be one hour at 37°C or overnight at 4°C (85,90). Optimal antibody binding time was found to be a minimum of thirty minutes (85). One hour has been used previously in our laboratory (90). Significant binding of conjugate occurs within thirty minutes (92). Therefore, these parameters were used in a standard MI ELISA. Although most of the parameters have been optimized, standardization of the antigen and conjugate concentrations was necessary.

Standardization of antigen concentration. A checkerboard titration (93) was performed with different dilutions of the positive and negative reference sera using the standard MI ELISA. A 1:100 antigen dilution containing 7.6 mcg/ml of protein was chosen. (Fig 8.) According to Clark and Adams (73), optimum coating of wells occurs with a protein concentration between 1 - 10 mcg/ml. The 1:100 dilution of AIBV gave maximum binding of antisera and showed the best comparison between high sensitivity and low non-specific binding.

Standardization of conjugate concentration. A checkerboard titration of the conjugate with positive and negative reference sera was run using the standard MI ELISA. A working dilution of 1:2,000 was chosen for the test due to an interest in economy. This dilution had a minimum amount of non-specific binding but high sensitivity. (Fig 9.)

Standardization of the reference serum dilutions. The titration of a known positive and negative antisera is depicted in Fig 10. The endpoint titer was 1:4092 for the known positive serum and less than 1:32 for the known negative serum. It was concluded that the specific dilution employed for the titration of the positive and negative reference sera would be enough to cover the complete range of the negative and positive end points expected in the test samples.

Substrate incubation time. The substrate incubation time was determined in the same manner as the MIDAS ELISA, except a reference antibody was used instead of the antigen.

Reproducibility of results. Repeated titrations of selected sera over a period of one month showed no significant variability in the end point titers.

Experiment 1. Fifteen chickens comprised the control group and twenty-one chickens comprised the infected group.

Clinical observations. Chickens exhibited respiratory signs of AIBV (1,7). Signs commenced on the third day postinfection and continued until the seventh day. No signs were evident from the fourteenth day onward. The control chickens showed no signs of infection throughout the study.

Post-mortem examination. Chickens sacrificed and examined, showed cloudy air sacs; congested and hemorrhagic tracheas, with adherent mucous exudate; and congested and discolored

lungs.

Virus isolation. Table 1 shows virus isolation at different days postinfection and at various passages in embryonated chicken eggs. The virus was detected in 80% of the tracheal swabs tested at day one, in 100% of the swabs tested at three and seven days, and in 20% of the swabs at day fourteen. No virus was detected in the control swabs.

The virus was detected in the feces of 40% of the swabs tested after one and seven days, 60% at three days, and 20% after day fourteen. Note that chicken no. 35 was late in showing signs of the virus and also persisted longer in shedding of the virus.

MIDAS ELISA. Table 2 shows the results of the detection of viral antigen in the trachea and fecal swabs of the infected chickens. These results were comparable with those of virus isolation. No virus was detected after twenty one days postinfection. The lungs from various chickens sacrificed throughout the study demonstrated the presence of virus from day three to day seven. No virus was detected in the lung after seven days.

MI ELISA. The immune response of the chickens is shown in Table 3. A rise in titer was detected after seven days and continued to increase up to twenty-one days. In three out of five chickens, there was a continued rise in titer up to the end of the study, while one of the remaining chickens decreased in titer and the other remained the same.

Experiment 2. Twenty chickens comprised the control group and twenty chickens comprised the infected group.

Clinical signs. Chickens exhibited signs of respiratory distress of AIBV infection (1,7). Signs commenced after the second day postinfection and continued until the fourteenth day. No signs were evident from the twentieth day onward. Control chickens showed no signs of infection throughout the study.

Post-mortem examination. Post-mortem examination of the sacrificed chickens revealed similar signs as described in Experiment 1.

Three chickens died after one week and two chickens died after two weeks. The chickens appeared dehydrated and emaciated.

Virus isolation. Table 4. shows that six out of six of the chickens infected contained virus in their throat swabs from day one to day fourteen. No virus was detected after day fourteen.

In the fecal swabs, virus was detected up to day fourteen in four out of the six infected chickens. No virus was detected after day fourteen.

No virus was isolated from the control chickens.

MIDAS ELISA. Table 5 shows the presence of virus in 83%, 100%, 33%, and 17% of the tracheal swabs after one, three to fourteen, twenty-one, and twenty-eight days post-infection respectively. These test results included only six infected

chickens. The chickens were reinfected after twenty-eight days, but, no virus was recovered.

In the fecal swabs, virus was recovered in 67% of the samples from day one to seven, in 83% after day fourteen, in 33% on day twenty-one, twenty-eight, and one day after reinfection. No virus was recovered at day seven after reinfection.

The lungs from various chickens sacrificed throughout the study demonstrated the presence of virus from day three to day fourteen. No virus was detected in the lungs after day fourteen.

MI ELISA. Table 6 shows a rise in antibody titer after seven days, reaching a maximum titer at twenty-one days, and declining after reinfection. After twenty-eight days, five out of eleven chickens showed a decline in titer, two of the remaining chickens showed a rise in titer, and the remaining four chickens remained the same. After reinfection: at seven days, two out of eleven chickens declined in antibody titer, one rose, and eight chickens remained at the same titer; at fourteen days, seven out of eleven chickens declined, two rose, and two remained at the same titer.

Cumulative results. Table 7. and Table 8. show the cumulative results of virus isolation in chicken embryos and by MIDAS ELISA respectively. All controls were

negative, while throat and fecal swabs of the infected chickens demonstrated the presence of virus. It appears that isolation of virus by chicken embryos or MIDAS ELISA were comparable.

Table 9. shows an increase in the mean log₂ titers of antibody after day seven in both Experiments 1 and 2. In Experiment 1, there was a peak antibody titer after fourteen days and the mean titers remained high until the end of the study. After day three, the mean titers showed a significant ($p = 0.02$) difference between the control and infected groups. In Experiment 2, there was a peak antibody titer at day fourteen followed by a slow decline in titer. All the results were statistically significant ($p = 0.001$).

Fig 11. illustrates the antibody response of the control and infected groups in both experiments graphically.

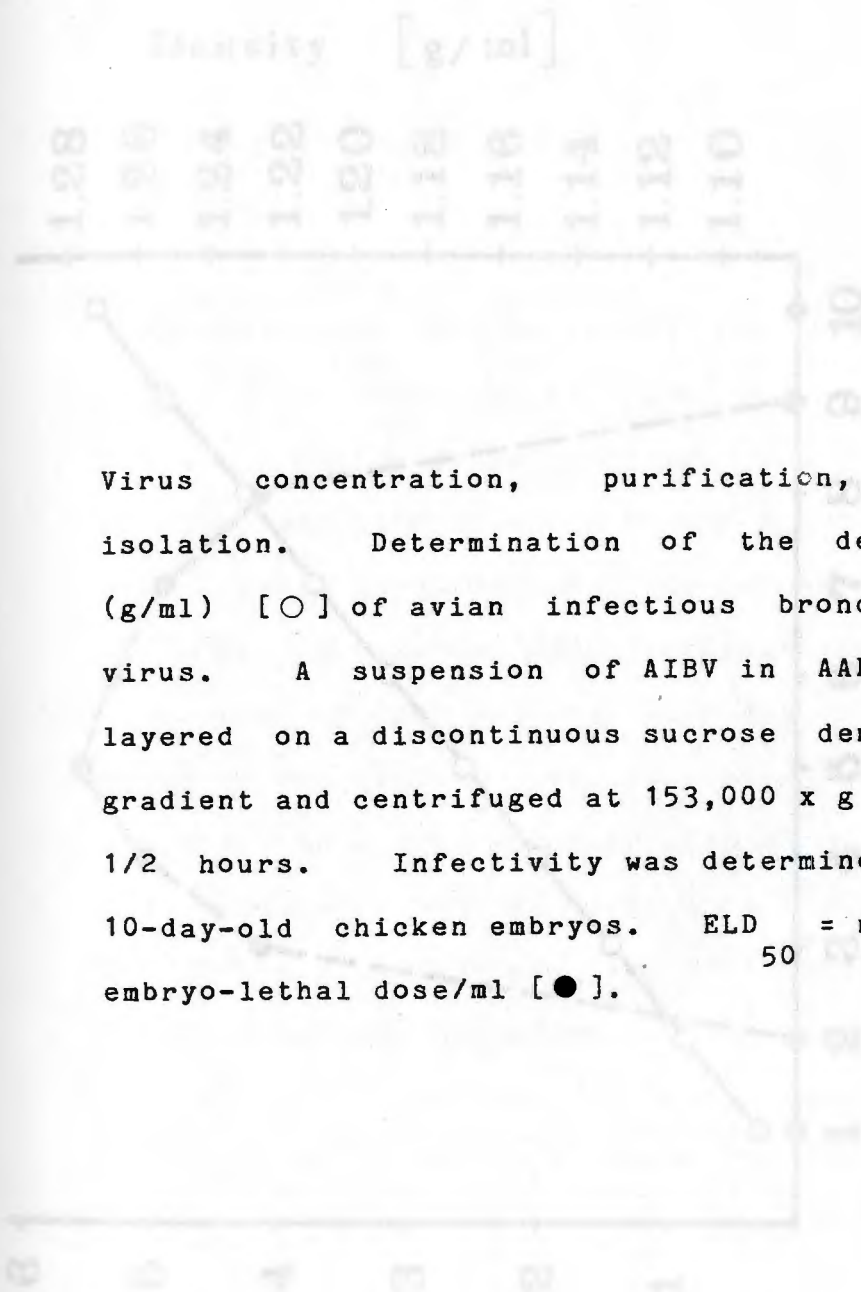


Figure 1.

Virus concentration, purification, and isolation. Determination of the density (g/ml) [○] of avian infectious bronchitis virus. A suspension of AIBV in AAF was layered on a discontinuous sucrose density-gradient and centrifuged at 153,000 x g for 3 1/2 hours. Infectivity was determined in 10-day-old chicken embryos. ELD₅₀ = median embryo-lethal dose/ml [●].

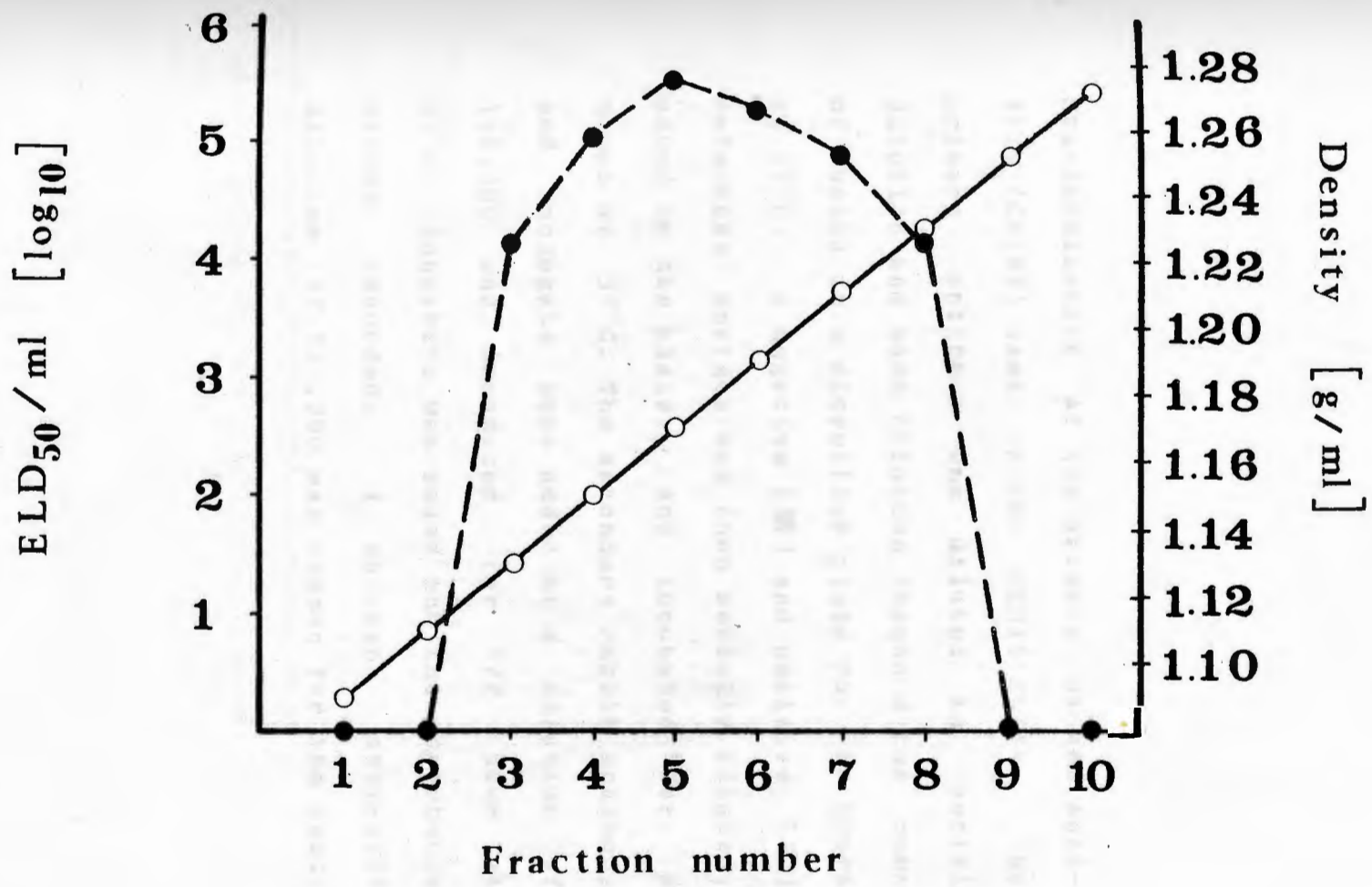


Figure 2.

Standardization of the primary chicken anti-AIBV (CAIBV) used in the MIDAS ELISA. The primary antibody was diluted in serial dilution and each dilution coated a two rows of wells in a microtiter plate for 2 hours at 37°C. A negative [■] and positive [□] reference antigen was then serially diluted, added to the plates, and incubated for 2 hours at 37°C. The secondary rabbit antibody and conjugate were added at a dilution of 1:2,000 and incubated for 1/2 hour at 37°C. Substrate was added and the absorbance values recorded. A chicken anti-AIBV dilution of 1:1,000 was chosen for the test.

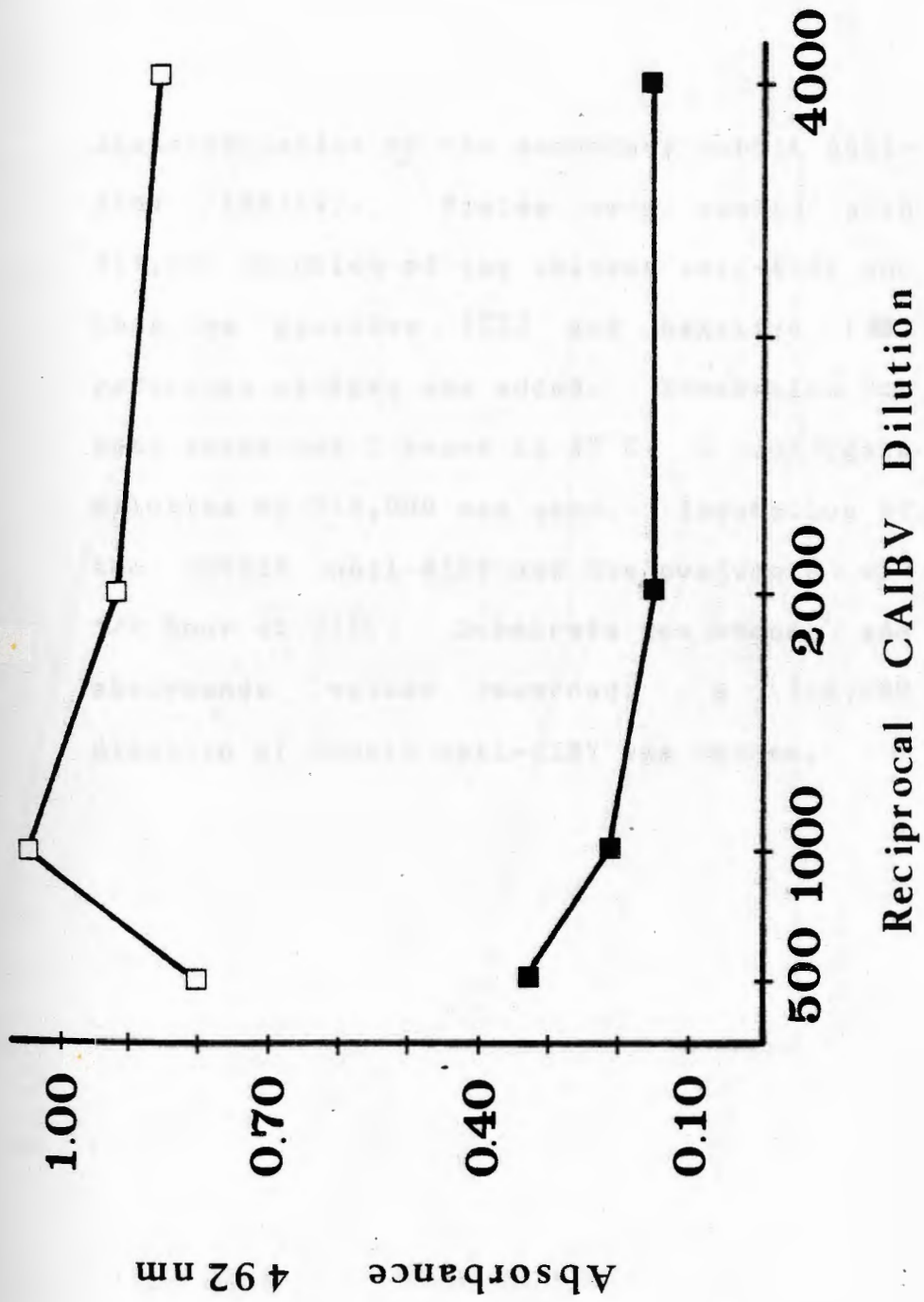
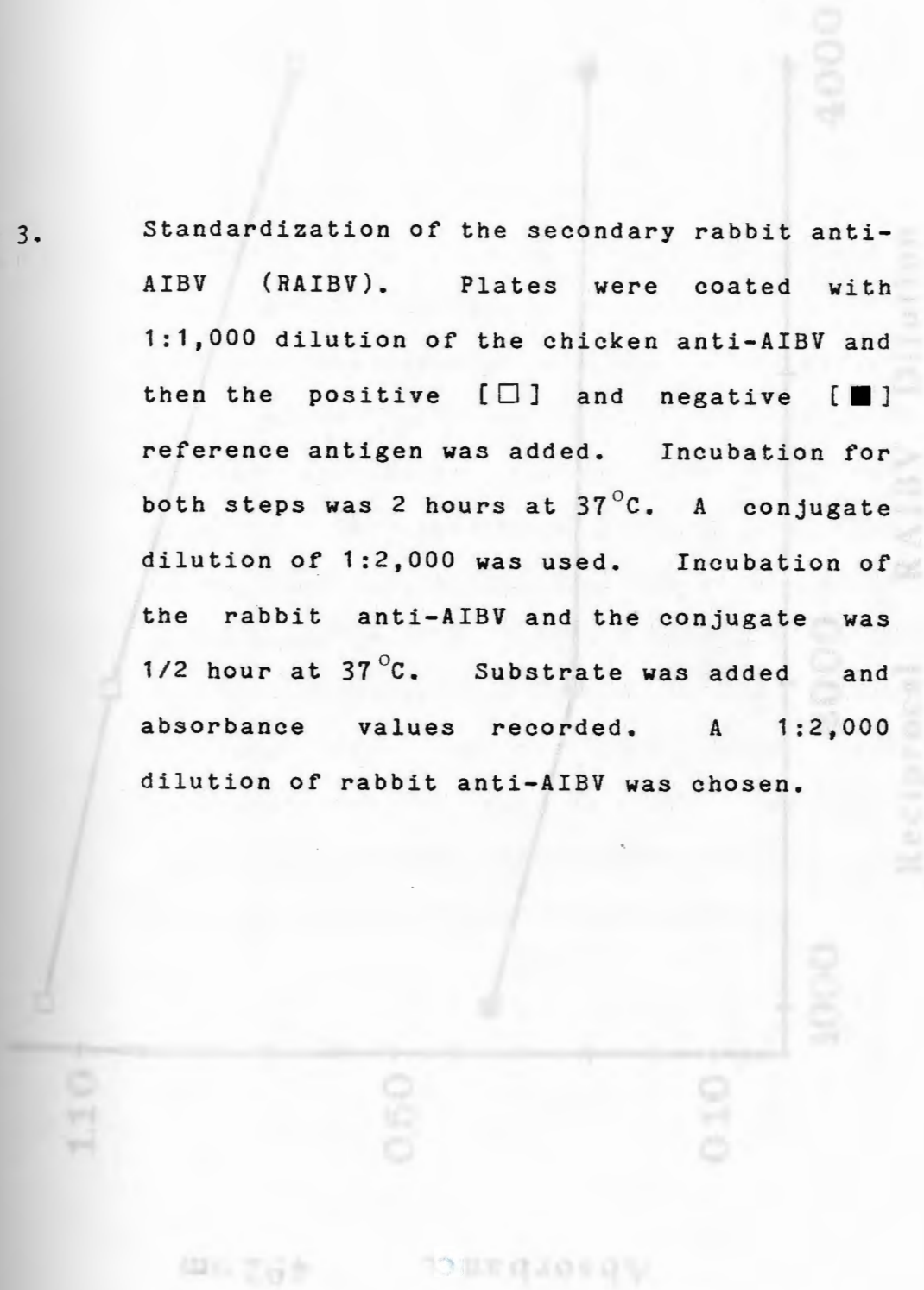


Figure 3.

Standardization of the secondary rabbit anti-AIBV (RAIBV). Plates were coated with 1:1,000 dilution of the chicken anti-AIBV and then the positive [□] and negative [■] reference antigen was added. Incubation for both steps was 2 hours at 37°C. A conjugate dilution of 1:2,000 was used. Incubation of the rabbit anti-AIBV and the conjugate was 1/2 hour at 37°C. Substrate was added and absorbance values recorded. A 1:2,000 dilution of rabbit anti-AIBV was chosen.



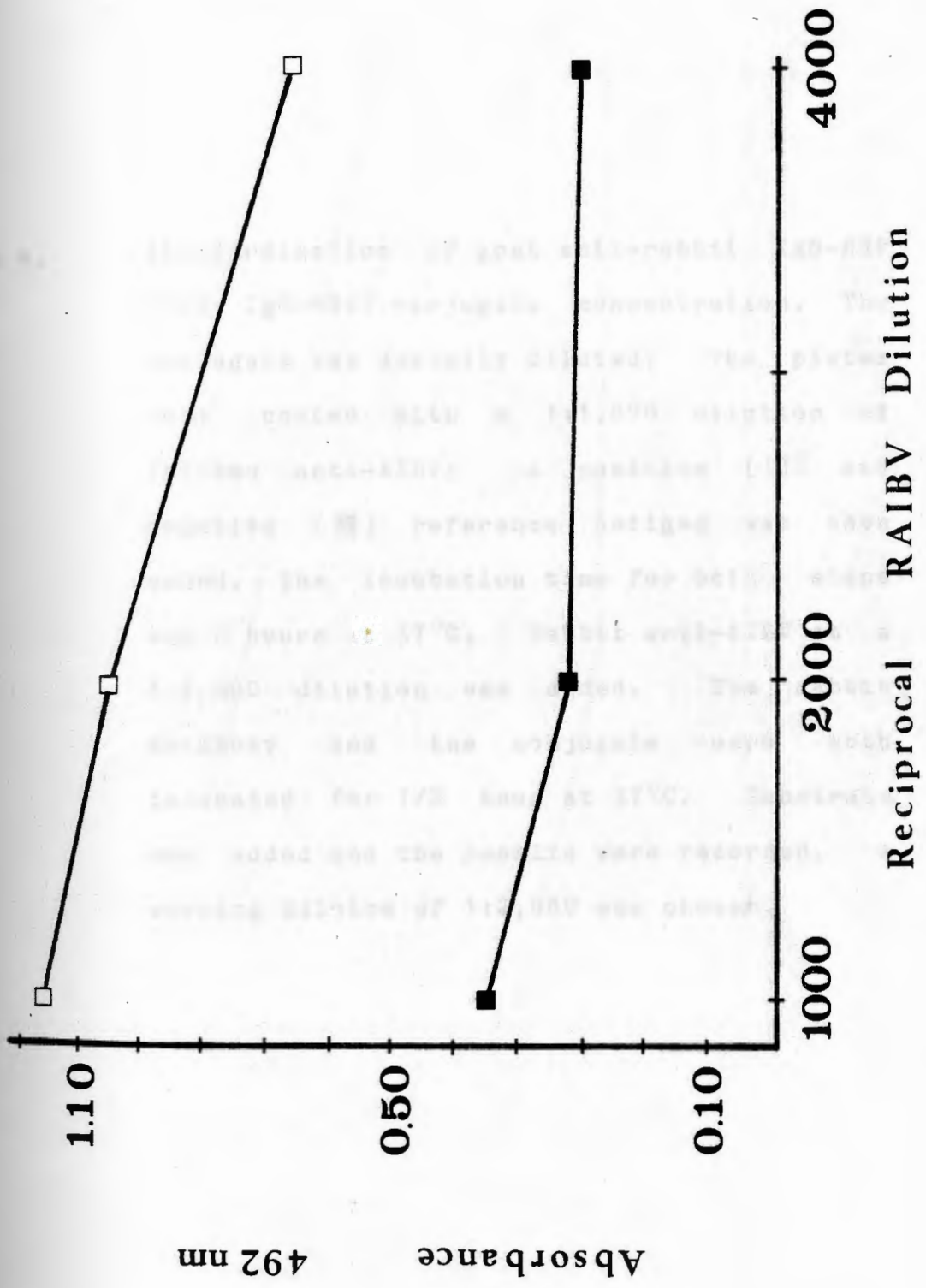
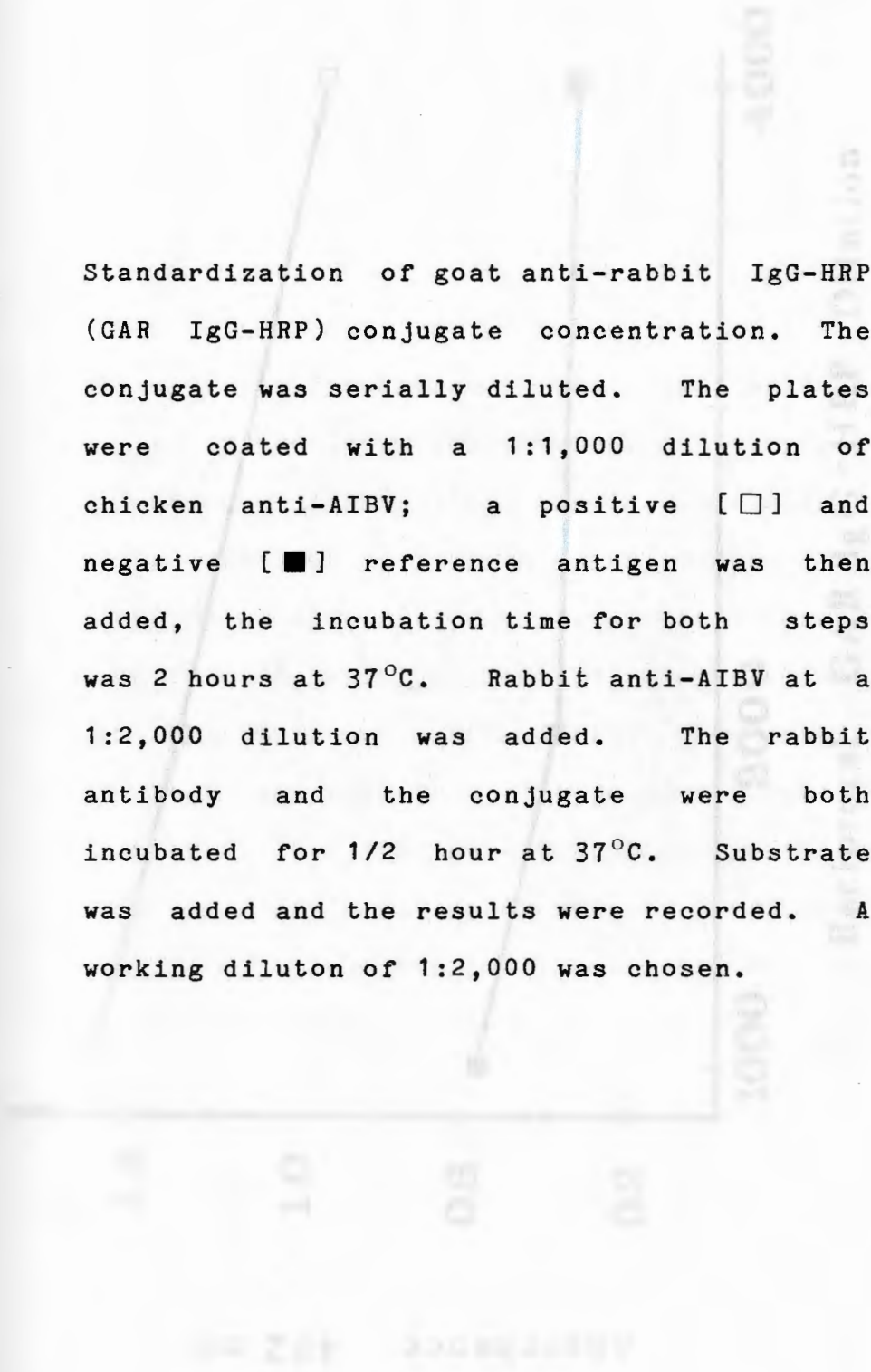


Figure 4.

Standardization of goat anti-rabbit IgG-HRP (GAR IgG-HRP) conjugate concentration. The conjugate was serially diluted. The plates were coated with a 1:1,000 dilution of chicken anti-AIBV; a positive [□] and negative [■] reference antigen was then added, the incubation time for both steps was 2 hours at 37°C. Rabbit anti-AIBV at a 1:2,000 dilution was added. The rabbit antibody and the conjugate were both incubated for 1/2 hour at 37°C. Substrate was added and the results were recorded. A working dilution of 1:2,000 was chosen.



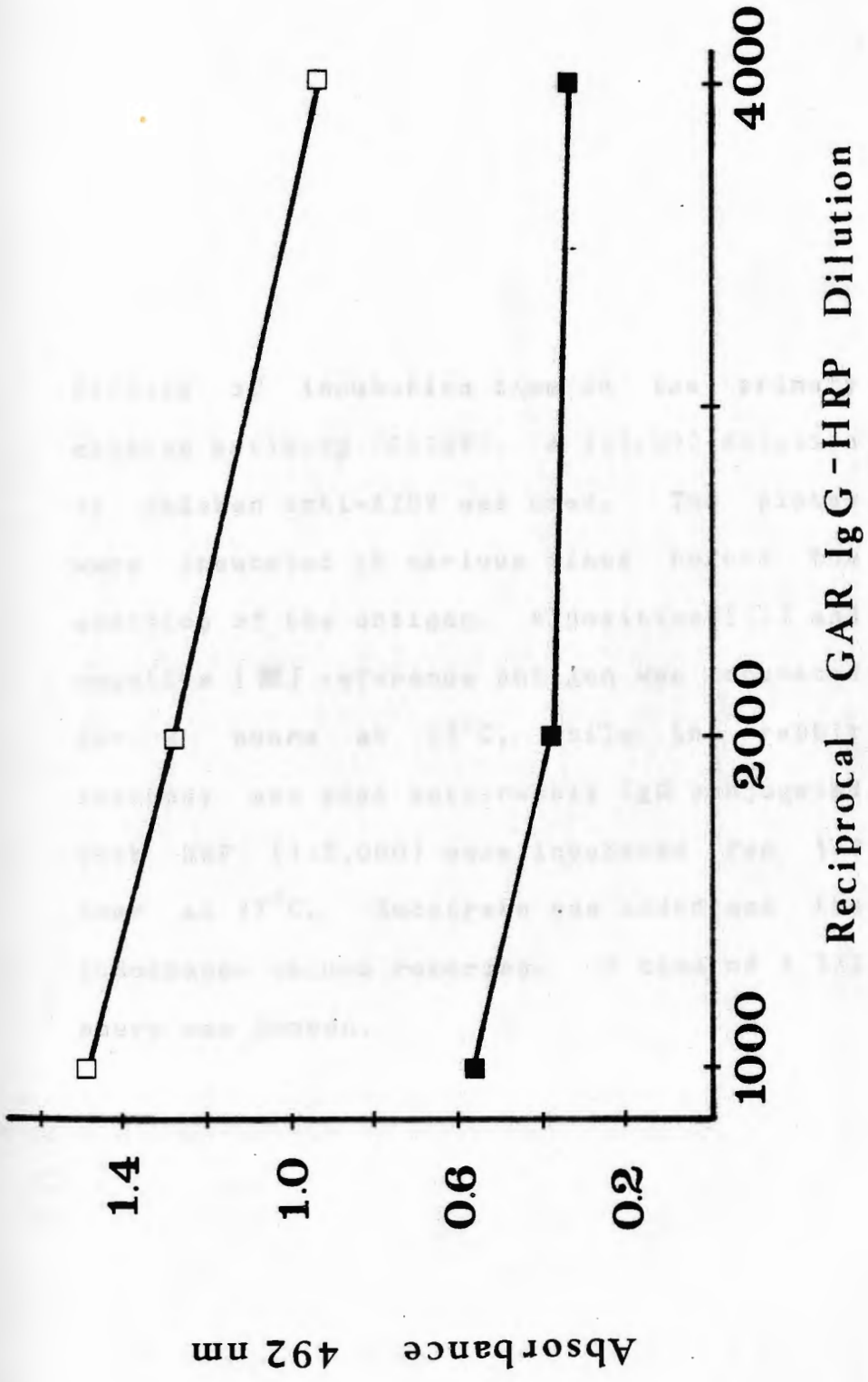
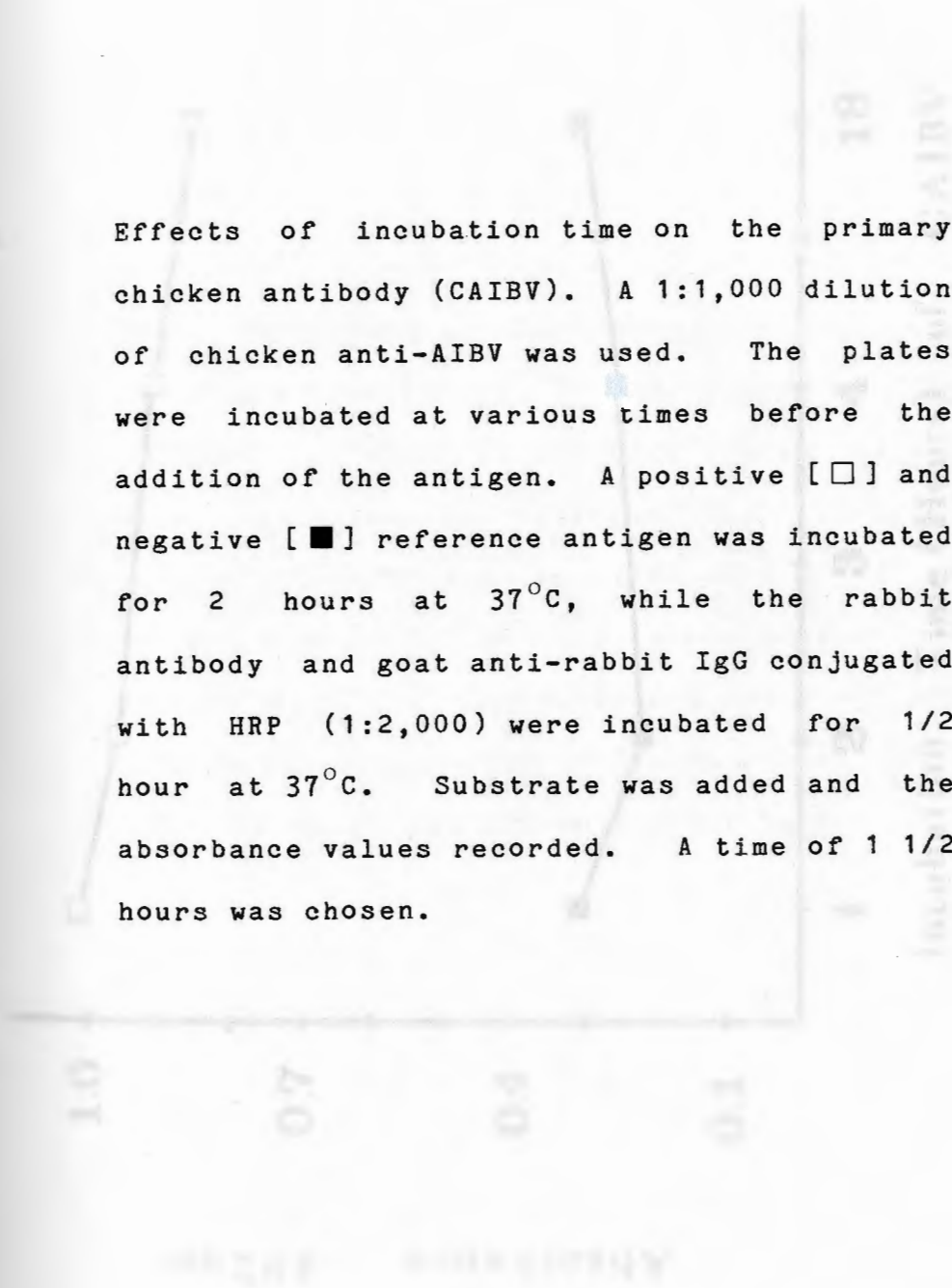


Figure 5.

Figure 5.

Effects of incubation time on the primary chicken antibody (CAIBV). A 1:1,000 dilution of chicken anti-AIBV was used. The plates were incubated at various times before the addition of the antigen. A positive [□] and negative [■] reference antigen was incubated for 2 hours at 37°C, while the rabbit antibody and goat anti-rabbit IgG conjugated with HRP (1:2,000) were incubated for 1/2 hour at 37°C. Substrate was added and the absorbance values recorded. A time of 1 1/2 hours was chosen.



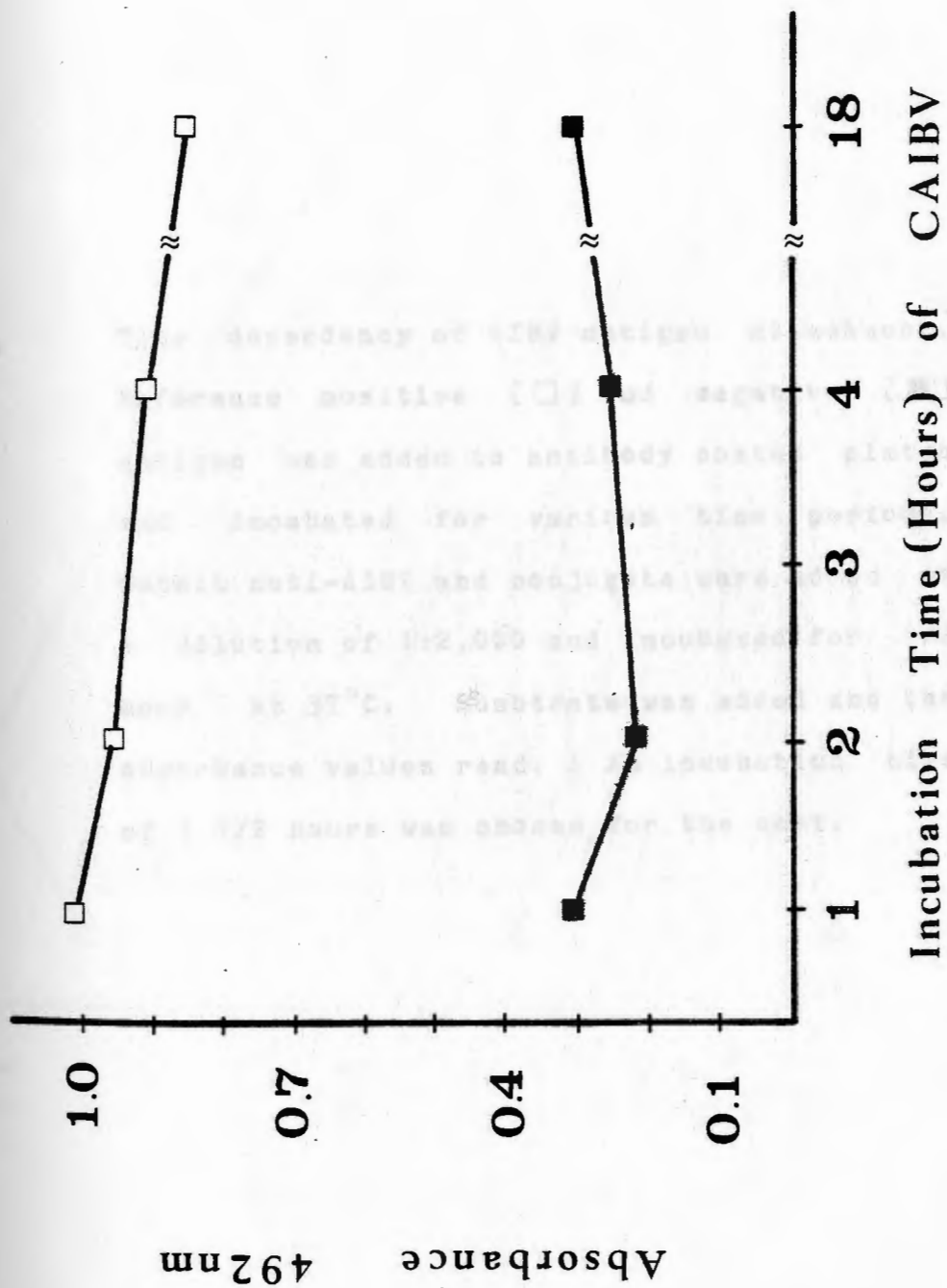
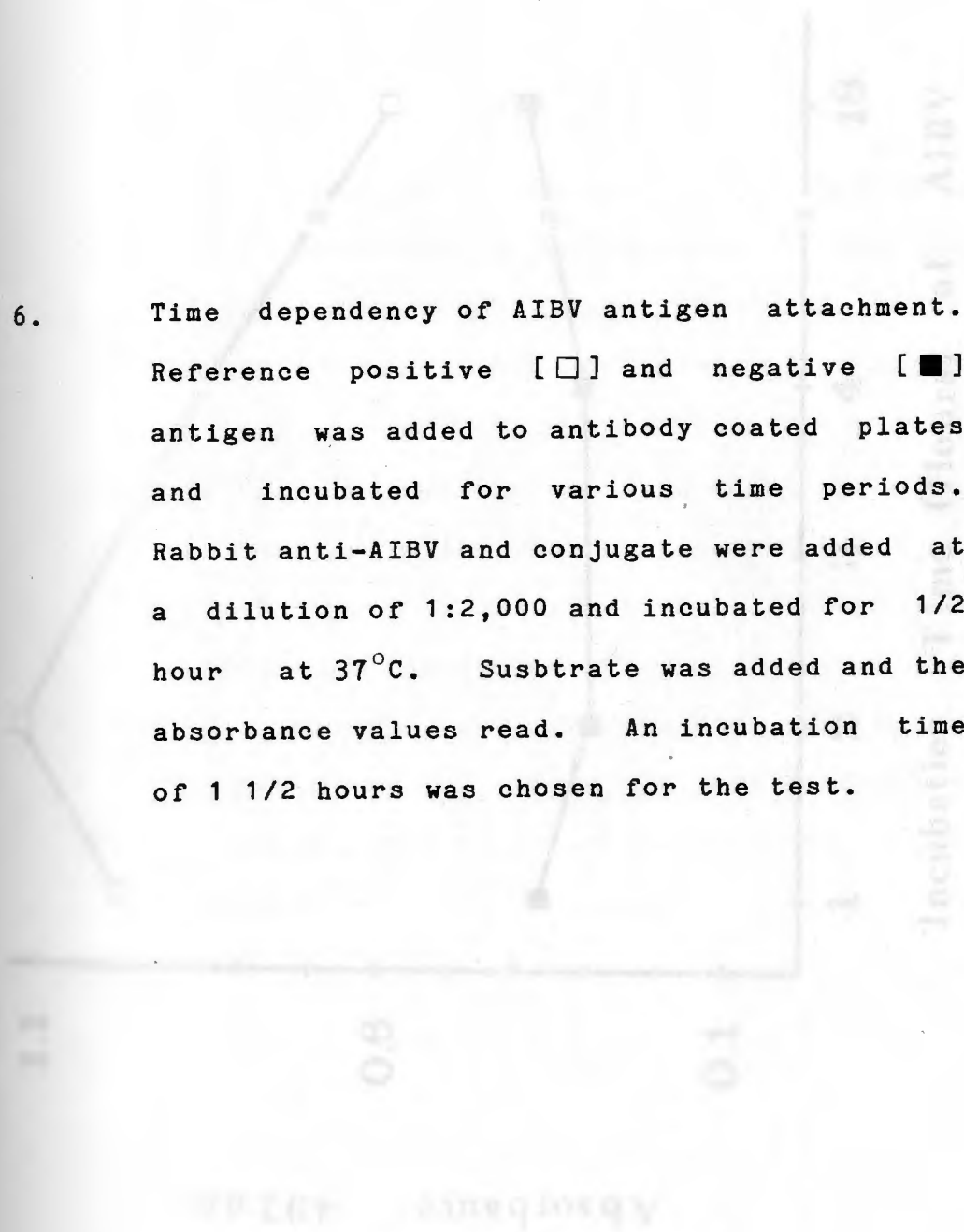


Figure 6.

Time dependency of AIBV antigen attachment. Reference positive [□] and negative [■] antigen was added to antibody coated plates and incubated for various time periods. Rabbit anti-AIBV and conjugate were added at a dilution of 1:2,000 and incubated for 1/2 hour at 37°C. Substrate was added and the absorbance values read. An incubation time of 1 1/2 hours was chosen for the test.



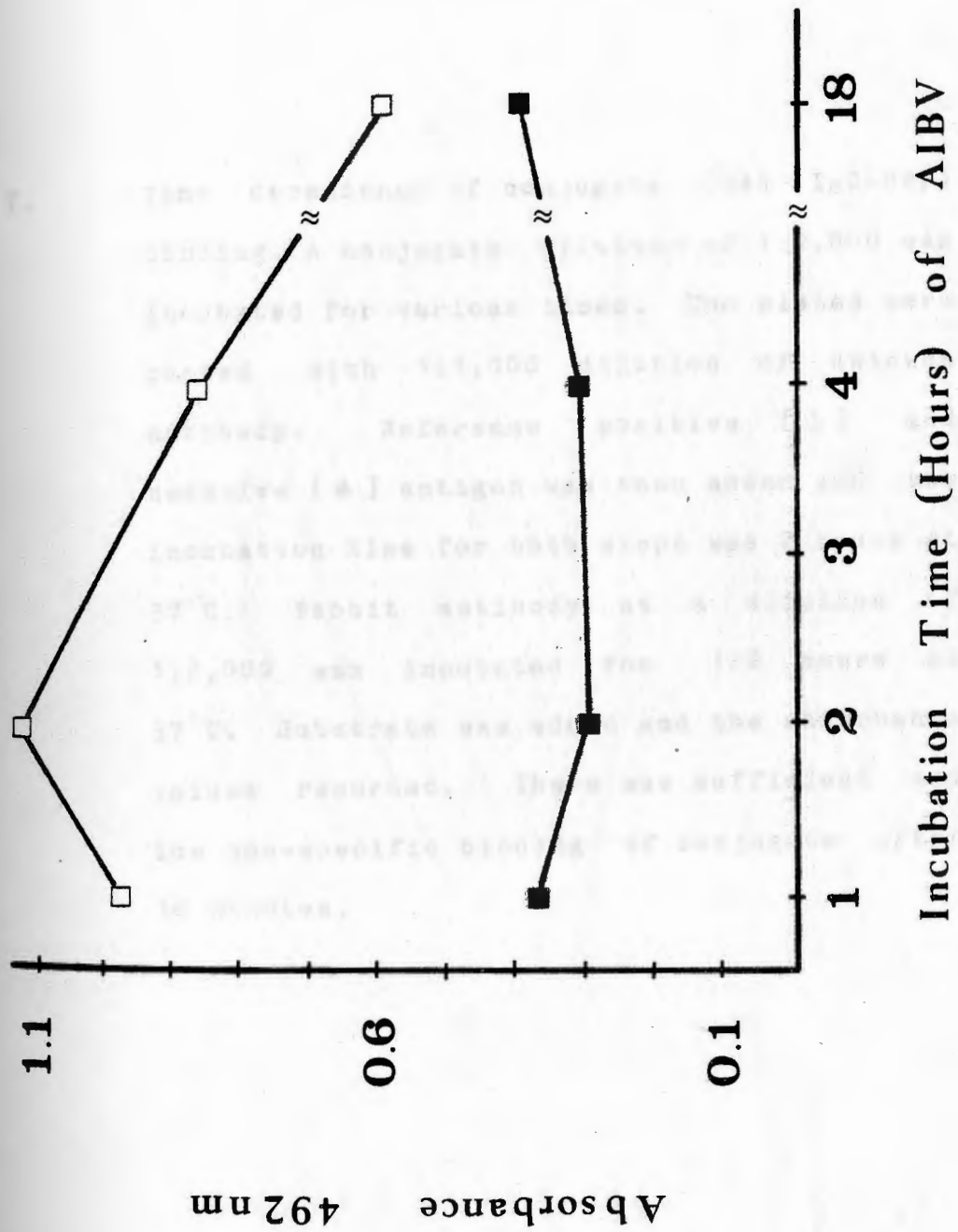
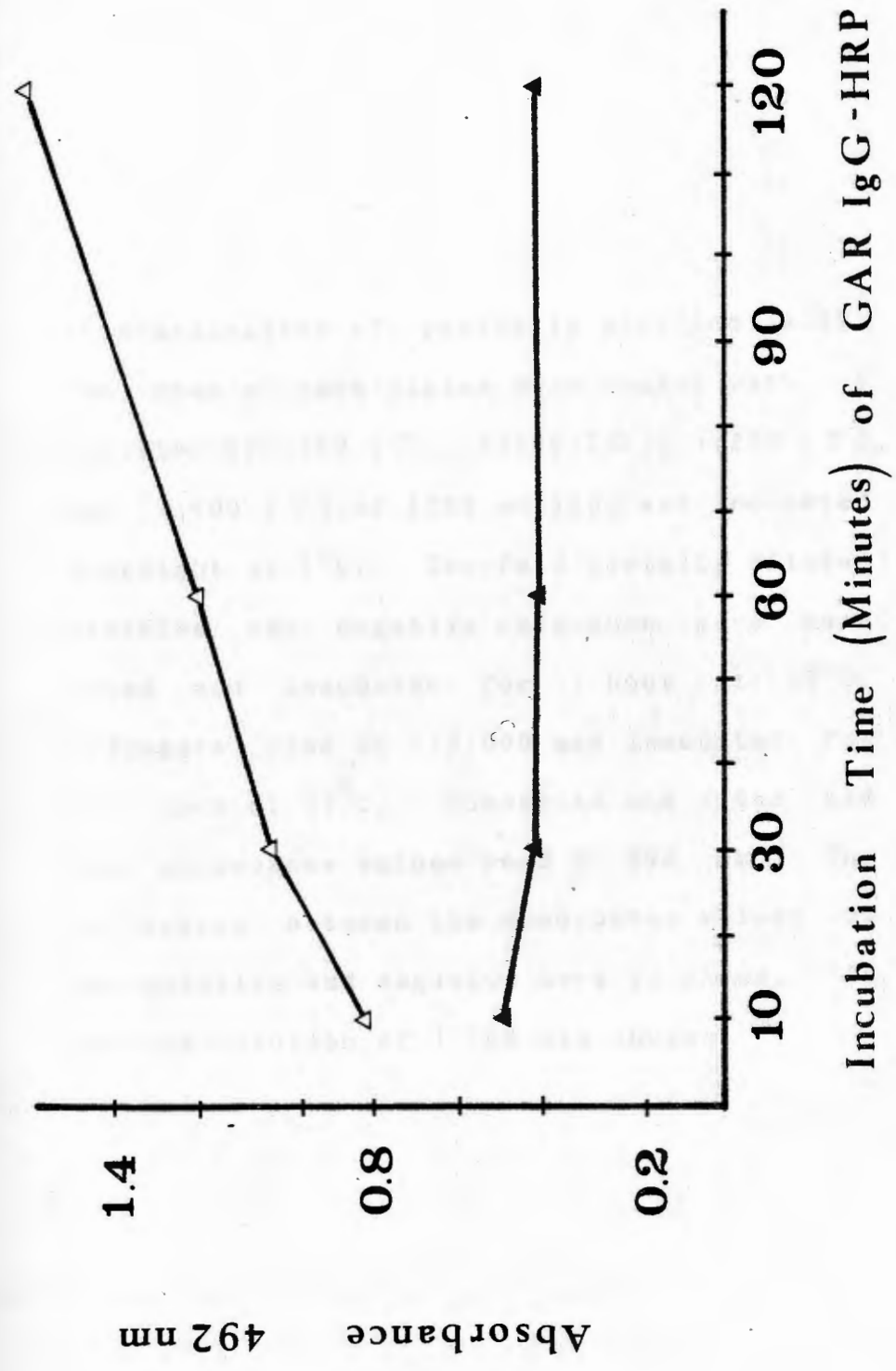


Figure 7.

Time dependency of conjugate (GAR IgG-HRP) binding. A conjugate dilution of 1:2,000 was incubated for various times. The plates were coated with 1:1,000 dilution of chicken antibody. Reference positive [Δ] and negative [\blacktriangle] antigen was then added and the incubation time for both steps was 2 hours at 37°C. Rabbit antibody at a dilution of 1:2,000 was incubated for 1/2 hours at 37°C. Substrate was added and the absorbance values recorded. There was sufficient and low non-specific binding of conjugate after 30 minutes.



Incubation Time (Minutes) of GAR IgG-HRP

Figure 8.

Standardization of partially purified AIBV. Two rows on each plates were coated with a dilution of 1:50 [○], 1:100 [△], 1:200 [▽], and 1:400 [◇] of AIBV antigen and incubated overnight at 4°C. Two-fold serially diluted positive and negative reference sera were added and incubated for 1 hour at 37°C. Conjugate used at 1:2,000 was incubated for 1/2 hour at 37°C. Substrate was added and the absorbance values read at 492 nm. The difference between the absorbance values of the positive and negative sera is shown. An antigen dilution of 1:100 was chosen.

Absorbance (492 nm Difference)
[Positive - negative reference]

Absorbance 492nm Difference
[positive - negative reference]

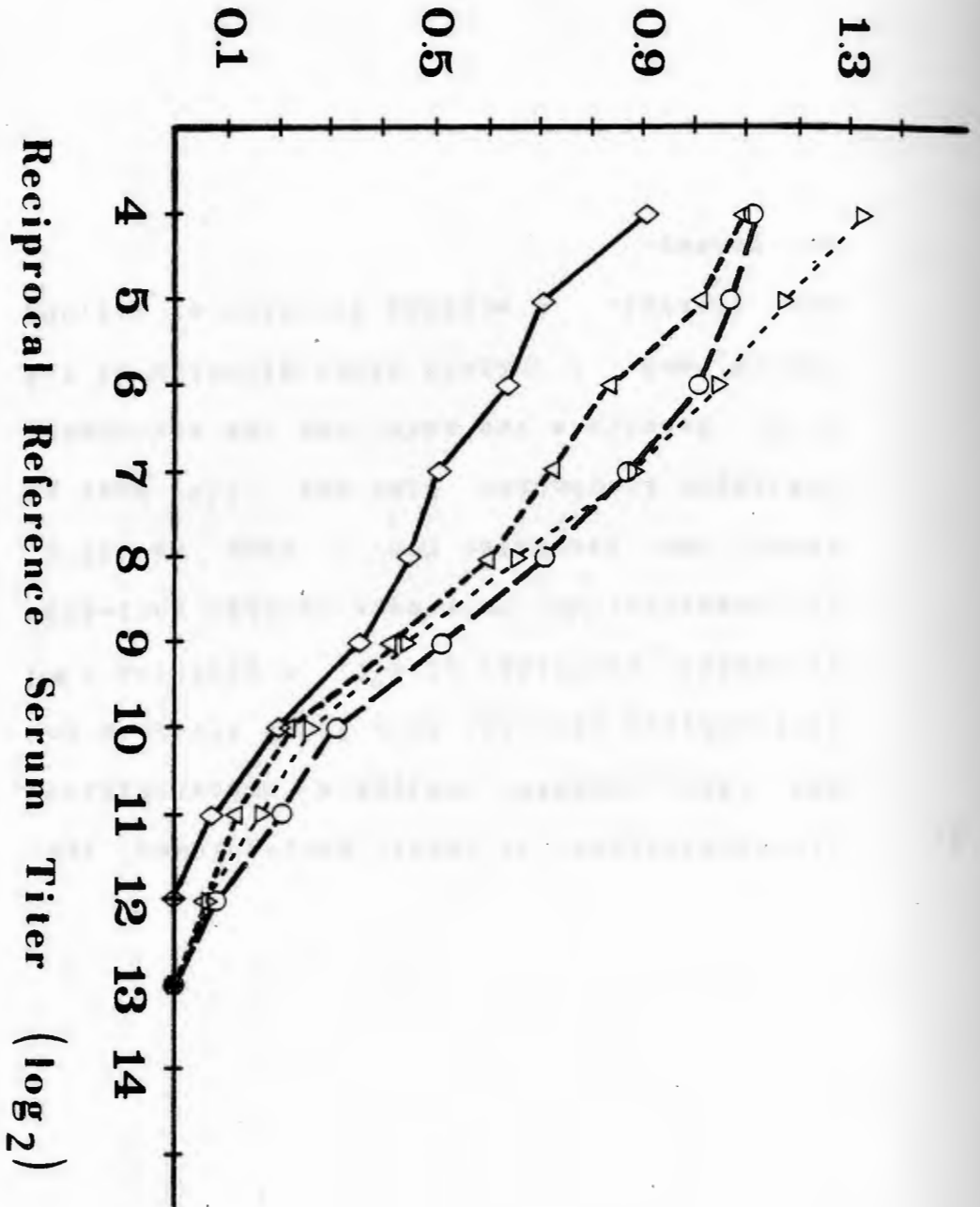
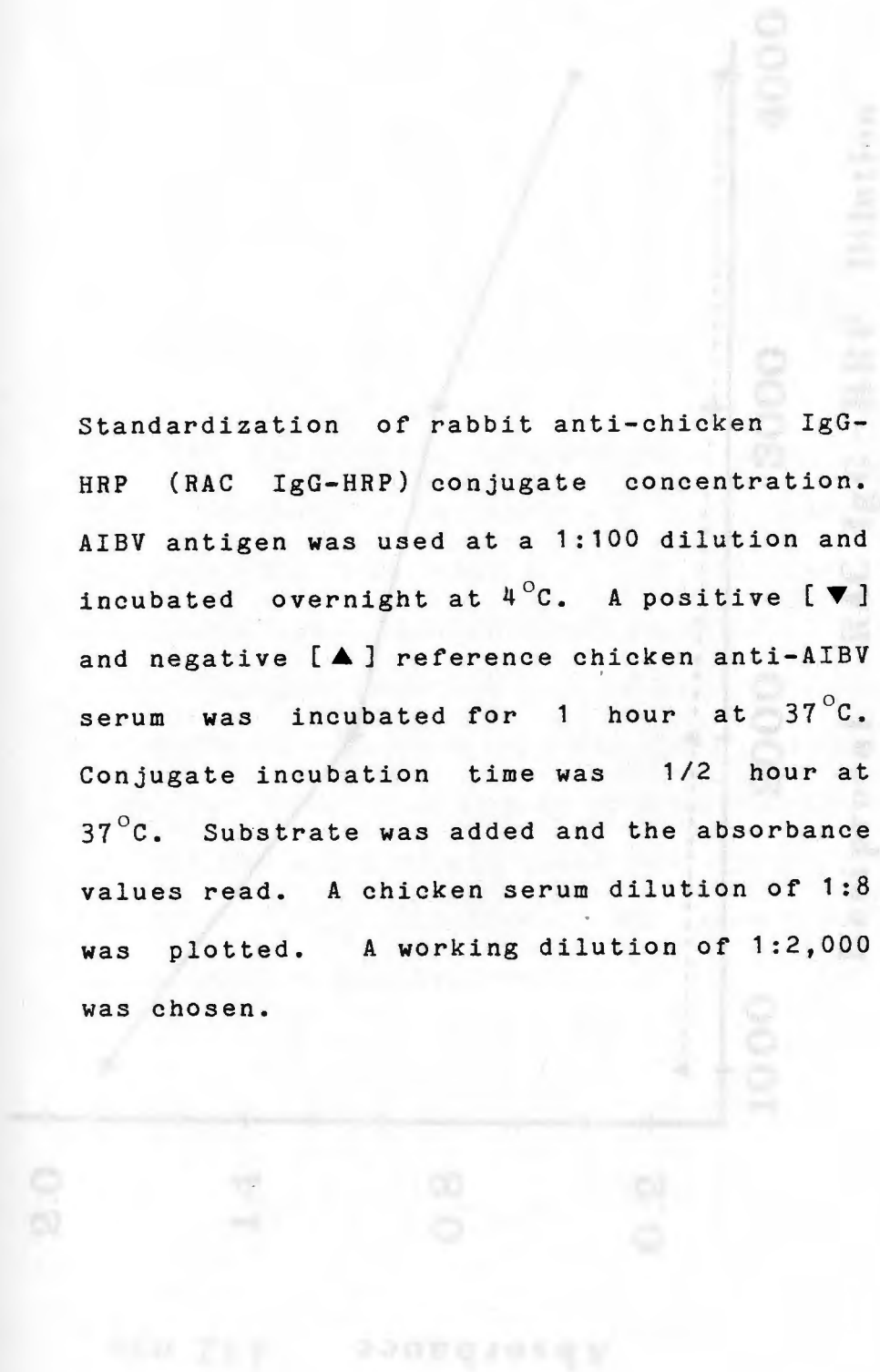


Figure 9.

Standardization of rabbit anti-chicken IgG-HRP (RAC IgG-HRP) conjugate concentration. AIBV antigen was used at a 1:100 dilution and incubated overnight at 4°C. A positive [▼] and negative [▲] reference chicken anti-AIBV serum was incubated for 1 hour at 37°C. Conjugate incubation time was 1/2 hour at 37°C. Substrate was added and the absorbance values read. A chicken serum dilution of 1:8 was plotted. A working dilution of 1:2,000 was chosen.



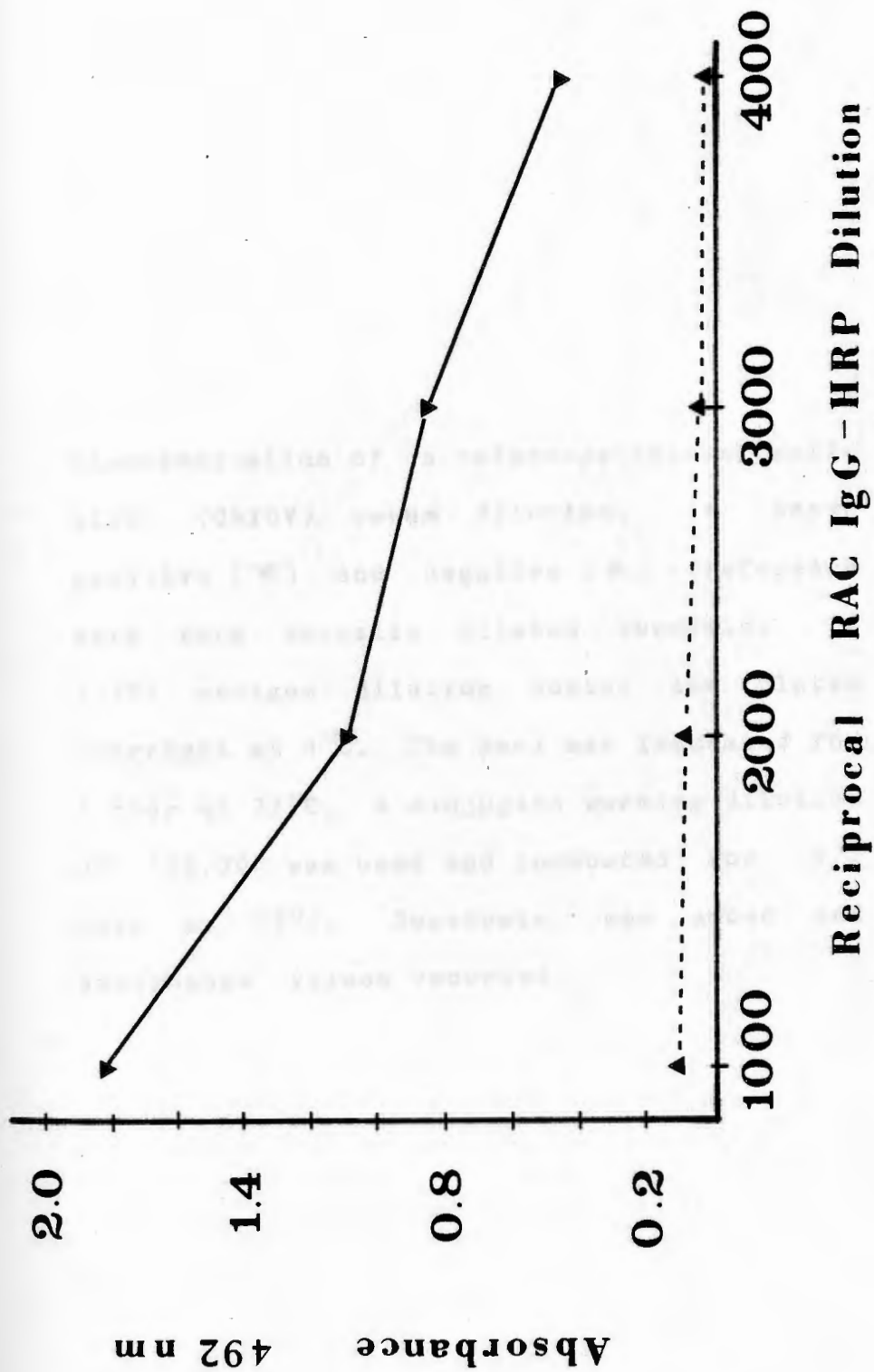
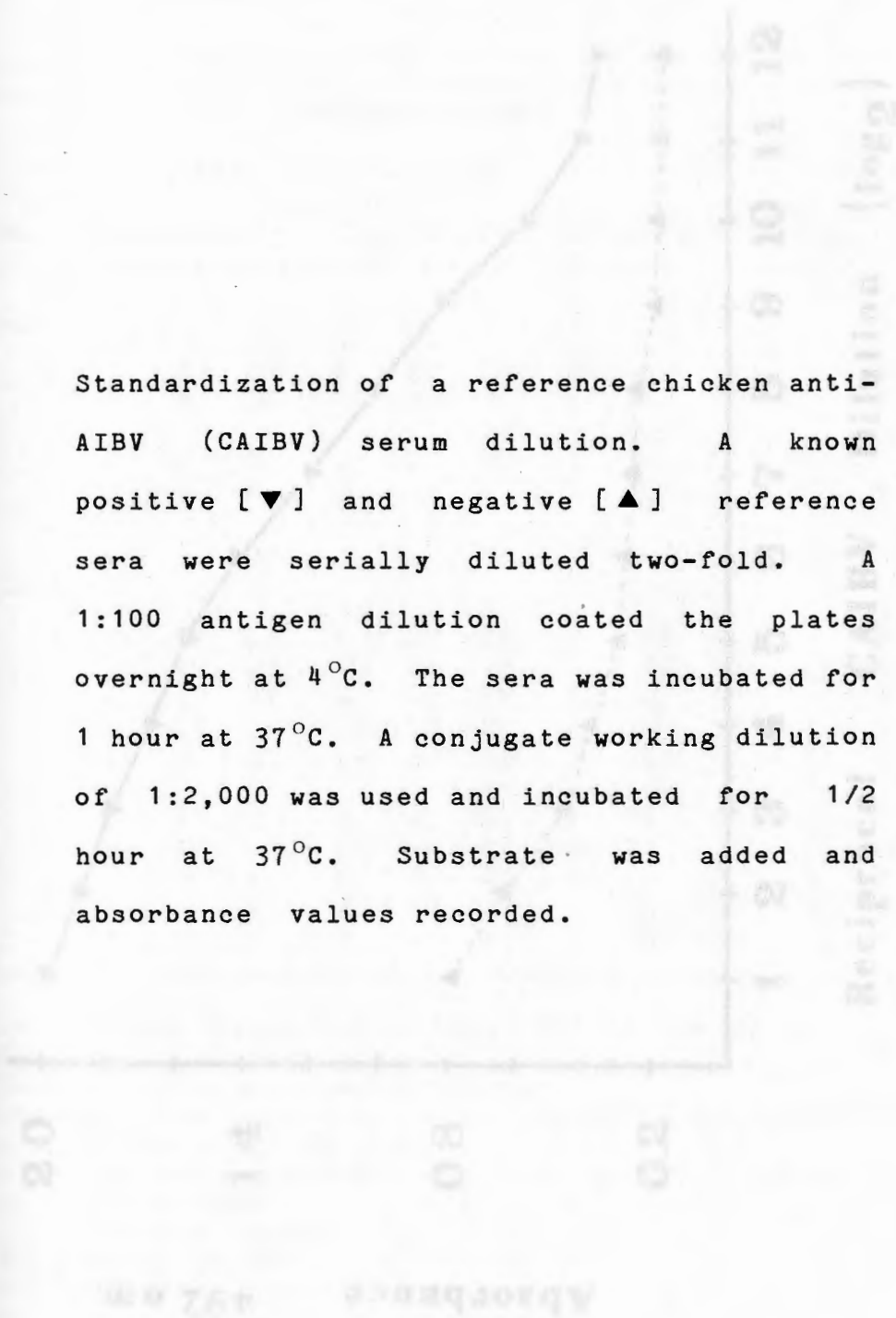


Figure 10.

Standardization of a reference chicken anti-AIBV (CAIBV) serum dilution. A known positive [\blacktriangledown] and negative [\blacktriangle] reference sera were serially diluted two-fold. A 1:100 antigen dilution coated the plates overnight at 4°C. The sera was incubated for 1 hour at 37°C. A conjugate working dilution of 1:2,000 was used and incubated for 1/2 hour at 37°C. Substrate was added and absorbance values recorded.



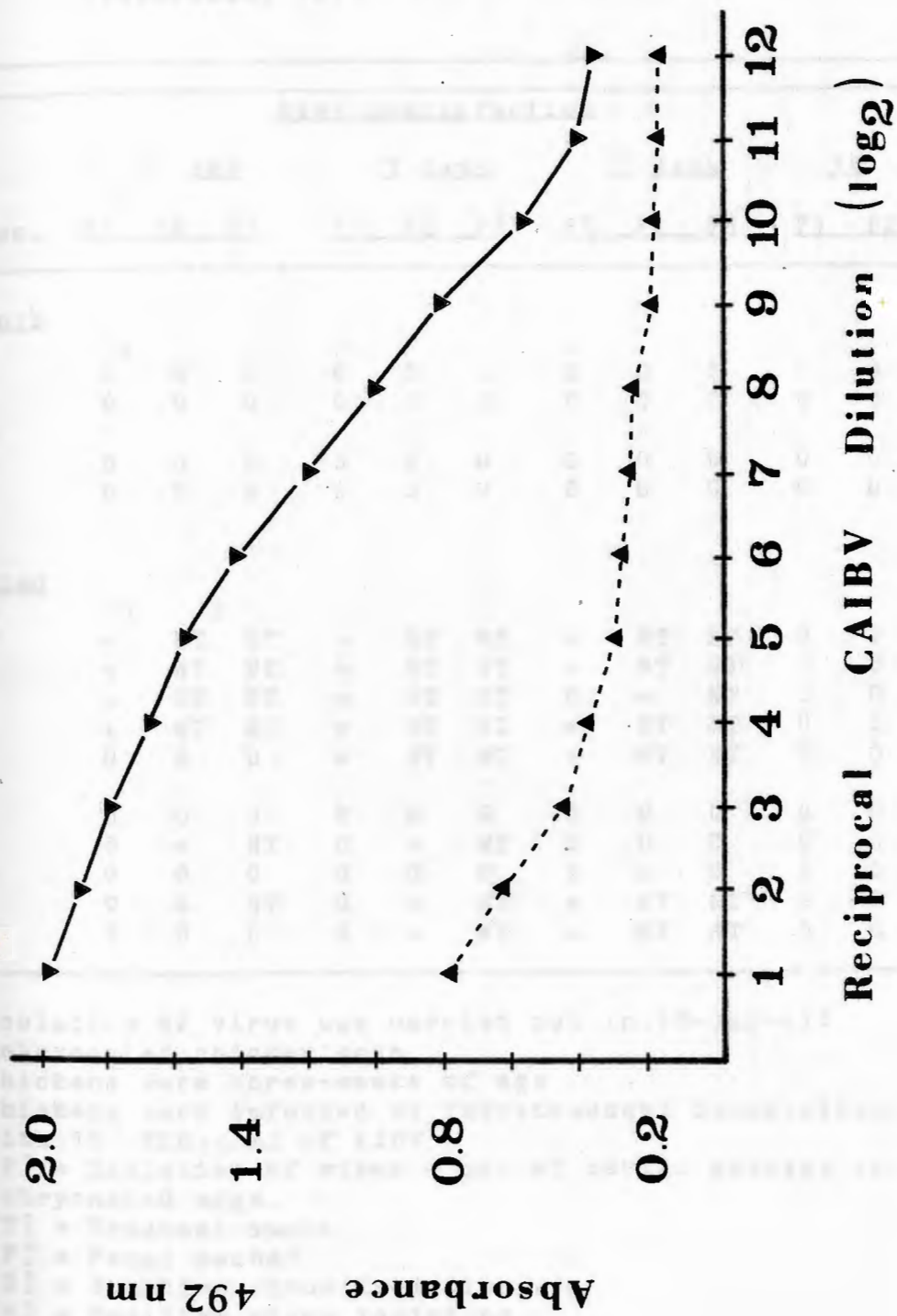


Table 1. Isolation^a of avian infectious bronchitis virus (AIBV) in the throat and feces of infected chickens^{b,c} by means of embryonated chicken eggs. (Experiment 1.)

Bird no.	Days postinfection											
	1 day			3 days			7 days			14 days		
	d											
	P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3
<u>Controls</u>												
e												
10 T	0	0	0	0	0	0	0	0	0	0	0	0
9 T	0	0	0	0	0	0	0	0	0	0	0	0
f												
10 F	0	0	0	0	0	0	0	0	0	0	0	0
9 F	0	0	0	0	0	0	0	0	0	0	0	0
<u>Infected</u>												
e												
h												
i												
31 T	+	NT	NT	+	NT	NT	+	NT	NT	0	0	0
32 T	+	NT	NT	+	NT	NT	+	NT	NT	0	0	0
33 T	+	NT	NT	+	NT	NT	0	+	NT	0	0	0
34 T	+	NT	NT	+	NT	NT	+	NT	NT	0	0	0
35 T	0	0	0	+	NT	NT	+	NT	NT	0	0	+
f												
31 F	0	0	0	0	0	0	0	0	0	0	0	0
32 F	0	+	NT	0	+	NT	0	0	0	0	0	0
33 F	0	0	0	0	0	0	0	0	0	0	0	0
34 F	0	+	NT	0	+	NT	+	NT	NT	0	0	0
35 F	0	0	0	0	+	NT	+	NT	NT	0	0	+

- a. Isolation of virus was carried out in 10-day-old embryonated chicken eggs
- b. Chickens were three-weeks of age
- c. Chickens were infected by intratracheal inoculation with 10^3 ELD₅₀/ml of AIBV
- d. [P] = Isolation of virus - no. of serial passage in embryonated eggs.
- e. [T] = Tracheal swabs
- f. [F] = Fecal swabs
- g. [0] = Negative virus isolation
- h. [+] = Positive virus isolation
- i. [NT] = Not tested

Table 2. Detection of AIBV antigen from the throat and feces of infected chickens^{a, b} by means of a modified indirect double antibody sandwich (MIDAS) ELISA. (Experiment 1.)

Bird no.	Days postinfection						
	1	3	5	7	14	21	28
<u>Controls</u>							
	^c	^e	^f				
6 T	NT	-	-	-	NT	-	-
7 T	-	-	-	-	-	-	-
8 T	-	NT	-	-	-	-	NT
9 T	-	-	NT	-	-	NT	-
10 T	-	-	-	NT	-	-	-
	^d						
6 F	NT	-	-	-	NT	-	-
7 F	-	-	-	-	-	-	-
8 F	-	NT	-	-	-	-	NT
9 F	-	-	NT	-	-	NT	-
10 F	-	-	-	NT	-	-	-
<u>Infected</u>							
	^c	^g					
31 T	+	+	+	+	-	-	-
32 T	+	+	+	+	-	-	-
33 T	+	+	+	+	-	-	-
34 T	+	+	+	+	-	-	-
35 T	-	+	+	+	+	-	-
	^d						
31 F	-	-	-	-	-	-	-
32 F	+	+	+	-	-	-	-
33 F	-	-	-	-	-	-	-
34 F	+	+	+	+	-	-	-
35 F	-	+	+	+	+	-	-

a. Chickens were infected by intratracheal inoculation with 10^3 ELD₅₀/ml of AIBV

b. Chickens were three-weeks of age

c. [T] = Tracheal swabs

d. [F] = Fecal swabs

e. [-] = Negative detection of antigen

f. [+] = Positive detection of antigen

g. [NT] = Not tested

Table 3. Antibody response in chickens^a following AIBV infection. (Experiment 1)^{b,c}

Bird no.	<u>Days postinfection</u>							
	^d P	1	3	5	7	14	21	28
<u>Control</u>								
	^e							
1	4				4	4	4	
2	16				16	16	16	
3	4				4	8	8	
4	8				8	8	16	
5	4				4	4	4	
6		8	8	8				8
7		8	8	8				4
8		4	4	4				4
9		8	8	8				16
10		4	4	4				16
<u>Infected</u>								
16	2	4			128			
17	4	4			64			
18	16	16			256			
19	4	4						
20	4	4			32			
21			8	8			32	
22			16	16			128	
23			8	32			256	
24			32	32			256	
25			8	8			64	
26		32	32					1024
27		4	4					32
28		16	16					32
29		16	16					32
30		32	32					256
31	4				64	512	1024	1024
32	8				128	1024	1024	2048
33	4				128	512	512	256
34	4				64	128	128	512
35	2				16	64	64	512

- a. Antibody was detected by the modified indirect enzyme-linked immunosorbent assay
 b. Chickens were infected by intratracheal inoculation with 10^3 ELD₅₀/ml of AIBV
 c. Chickens were three-weeks of age
 d. [P] = Pre-infection serum
 e. Antibody titer expressed as the reciprocal of serum dilution

Table 4. Isolation^a of AIBV in the throat and feces of infected chickens^{b,c} by means of embryonated chicken eggs. (Experiment 2.)

Bird no.	<u>Days postinfection</u>											
	<u>1 day</u>			<u>7 days</u>			<u>14 days</u>			<u>21 days</u>		
	<u>d</u> P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3
<u>Control Birds</u>												
^e 10 T	^g 0	0	0	0	0	0	0	0	0	0	0	0
9 T	0	0	0	0	0	0	0	0	0	0	0	0
6 T	0	0	0	0	0	0	0	0	0	0	0	0
5 T	0	0	0	0	0	0	0	0	0	0	0	0
^f 10 F	0	0	0	0	0	0	0	0	0	0	0	0
9 F	0	0	0	0	0	0	0	0	0	0	0	0
<u>Infected Birds</u>												
^e 25 T	^h +	ⁱ NT	NT	+	NT	NT	+	NT	NT	0	0	0
26 T	+	NT	NT	+	NT	NT	0	+	NT	0	0	0
27 T	+	NT	NT	+	NT	NT	0	+	NT	0	0	0
29 T	+	NT	NT	+	NT	NT	0	+	NT	0	0	0
33 T	+	NT	NT	+	NT	NT	+	+	NT	0	0	0
35 T	+	NT	NT	0	+	NT	0	+	NT	0	0	0
^f 25 F	+	+	NT	+	+	NT	+	+	NT	0	0	0
26 F	0	0	0	0	0	0	0	0	0	0	0	0
27 F	0	+	NT	+	+	NT	0	+	NT	0	0	0
29 F	+	+	NT	+	+	NT	0	+	NT	0	0	0
33 F	0	0	0	0	0	0	0	0	0	0	0	0
35 F	0	+	NT	0	+	NT	0	0	+	D ^j	NT	NT

- a. Isolation of virus was carried out in 10-day-old embryonated eggs
- b. Chickens were three-weeks of age
- c. Chickens were infected by intratracheal inoculation with 10^3 ELD₅₀/ml
- d. [P] = Isolation of virus - no. of serial passage in embryonated eggs
- e. [T] = Tracheal swabs
- f. [F] = Fecal swabs
- g. [0] = Negative virus isolation
- h. [+] = Positive virus isolation
- i. [NT] = Not tested
- j. [D] = Death of chicken during testing

Table 5. Detection of AIBV antigen from the throat and feces of infected chickens^{a,b} by means of a modified indirect double antibody sandwich (MIDAS) ELISA. (Experiment 2.)

Bird no.	<u>Days postinfection</u>								
	1	3	5	7	14	21	28 ^c	29	35
<u>Controls</u>									
	^d	^f						^g	
5 T	-	-	-	-	-	-	-	NT	NT
6 T	-	-	-	-	-	-	-	NT	NT
7 T	-	-	-	-	-	-	-	NT	NT
9 T	-	-	-	-	-	-	-	-	-
10 T	-	-	-	-	-	-	-	-	-
	^e								
5 F	-	-	-	-	-	-	-	NT	NT
6 F	-	-	-	-	-	-	-	NT	NT
7 F	-	-	-	-	-	-	-	NT	NT
9 F	-	-	-	-	-	-	-	-	-
10 F	-	-	-	-	-	-	-	-	-
<u>Infected</u>									
	^d	^h							
25 T	+	+	+	+	+	+	-	-	-
26 T	-	+	+	+	+	-	-	-	-
27 T	+	+	+	+	+	-	-	-	-
29 T	+	+	+	+	+	-	-	-	-
33 T	+	+	+	+	+	-	-	-	-
34 T	NT	NT	NT	NT	NT	+	+	-	-
35 T	+	+	+	+	+	D ⁱ	D	D	D
	^e								
25 F	+	+	+	+	+	-	-	-	-
26 F	-	-	-	-	+	-	-	-	-
27 F	+	+	+	+	+	+	+	+	-
29 F	+	+	+	+	+	+	+	+	-
33 F	-	-	-	-	-	-	-	-	-
34 F	NT	NT	NT	NT	NT	+	-	-	-
35 F	+	+	+	+	+	D	D	D	D

- a. Chickens were infected by intratracheal inoculation with 10^3 ELD₅₀/ml
- b. Chickens were three-weeks of age
- c. Reinfection of chickens after twenty-eight days postinfection intraocularly with 10^3 ELD₅₀/ml of AIBV
- d. [T] = Tracheal swabs
- e. [F] = Fecal swabs
- f. [-] = Negative detection of antigen
- g. [NT] = Not tested
- h. [+] = Positive detection of antigen
- i. [D] = Death of chicken

Table 6. Antibody^a response in chickens^{b,c} following AIBV infection. (Experiment 2.)

Bird no.	P ^d	Days postinfection					
		7	14	21	28 ^e	35	49
<u>Control</u>							
	f						
3	2	2	2	2	2	4	4
5	2	4	4	8	8	8	8
6	2	2	4	4	4	4	4
7	2	2	4	4	4	4	4
8	2	2	2	2	4	2	2
9	2	4	4	8	8	8	8
10	2	2	2	4	4	4	4
<u>Infected</u>							
	f						
22	4	32	2048	2048	1024	1024	512
23	4	16	2048	1024	512	512	512
24	4	64	512	512	1024	1024	512
25	8	16	1024	2048	1024	512	256
26	8	32	1024	1024	1024	512	256
27	4	64	2048	1024	1024	1024	512
29	8	64	512	512	1024	1024	2048
31	8	32	2048	1024	512	512	512
33	8	256	2048	2048	1024	1024	2048
34	2	64	64	64	64	64	512
35	16	512	1024	D ^g	-	-	-
41	2	32	2048	512	512	1024	512

- a. Antibody was detected by the modified indirect enzyme-linked immunosorbent assay
- b. Chickens were infected by intratracheal inoculation with 10^3 ELD₅₀/ml of AIBV
- c. Chickens were three-weeks of age
- d. [P] = Pre-infection serum
- e. Reinfection of the chickens after twenty-eight days postinfection intraocularly with 10^3 ELD₅₀/ml of AIBV
- f. Antibody titer expressed as the reciprocal of serum dilution
- g. [D] = Death of chicken

Table 7. Isolation^a of AIBV in the throat and feces of infected chickens^{b,c} by means of embryonated chicken eggs. Cumulative results of Experiment 1 and Experiment 2.

		<u>Days postinfection</u>				
		1	3	7	14	21
<u>Exp. no. 1</u>						
Control group						
d	f					g
T	0/2	0/2	0/2	0/2	0/2	NT
F ^e	0/2	0/2	0/2	0/2	0/2	NT
Infected group						
T	4/5	5/5	5/5	1/5	NT	
F	2/5	3/5	2/5	1/5	NT	
<u>Exp. no. 2</u>						
Control group						
T	0/4	NT	0/4	0/4	0/4	
F	0/2	NT	0/2	0/2	0/2	
Infected group						
T	6/6	NT	6/6	6/6	0/6	
F	4/6	NT	4/6	4/6	0/5	

- a. Isolation of virus was carried out in 10-day-old embryonated chicken eggs
- b. Chickens were three-weeks of age
- c. Chickens were infected by intratracheal inoculation with 10^3 ELD₅₀/ml of AIBV
- d. [T] = Tracheal swabs
- e. [F] = Fecal swabs
- f. number of positive isolations/ number of total tested
- g. [NT]= Not tested

Table 8. Detection of AIBV antigen from the throat and feces of infected chickens^{a,b} by means of a MIDAS ELISA. Cumulative results from Experiment 1 and Experiment 2.

		<u>Days postinfection</u>						
		1	3	5	7	14	21	28
Exp. no. 1								
Control group								
	^c							
T	^e	0/4	0/4	0/4	0/4	0/4	0/4	0/4
F	^d	0/4	0/4	0/4	0/4	0/4	0/4	0/4
Infected group								
T		4/5	5/5	5/5	5/5	1/5	0/5	0/5
F		2/5	3/5	3/5	2/5	1/5	0/5	0/5
Exp. no. 2								
Control group								
T		0/5	0/5	0/5	0/5	0/5	0/5	0/5
F		0/5	0/5	0/5	0/5	0/5	0/5	0/5
Infected group								
T		5/6	6/6	6/6	6/6	6/6	2/6	1/6
F		4/6	4/6	4/6	4/6	5/6	2/6	2/6

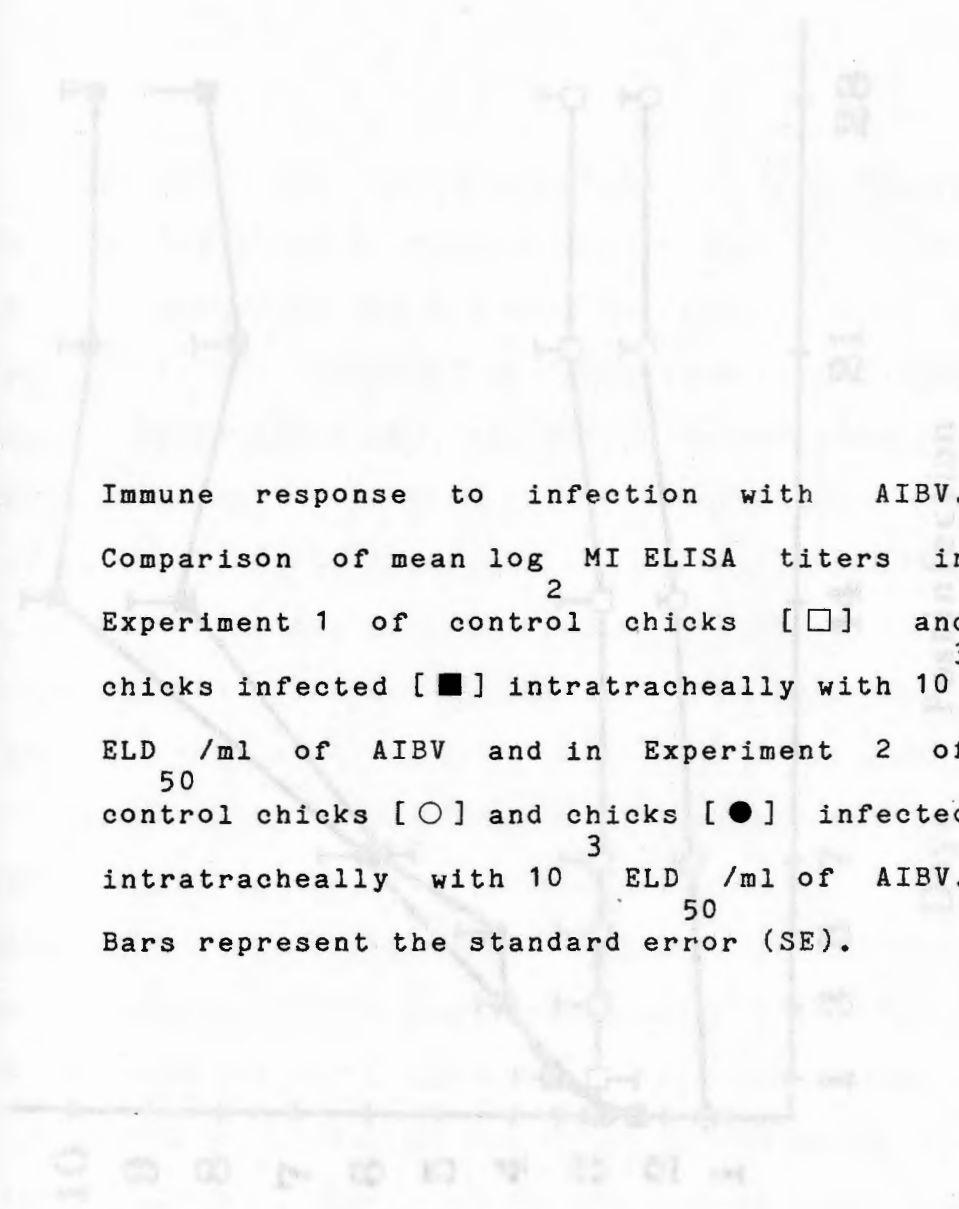
- a. Chickens were infected by intratracheal inoculation with 10^3 ELD₅₀/ml of AIBV
 b. Chickens were three-weeks of age
 c. [T] = Tracheal swabs
 d. [F] = Fecal swabs
 e. number of positive detections / number of total tested

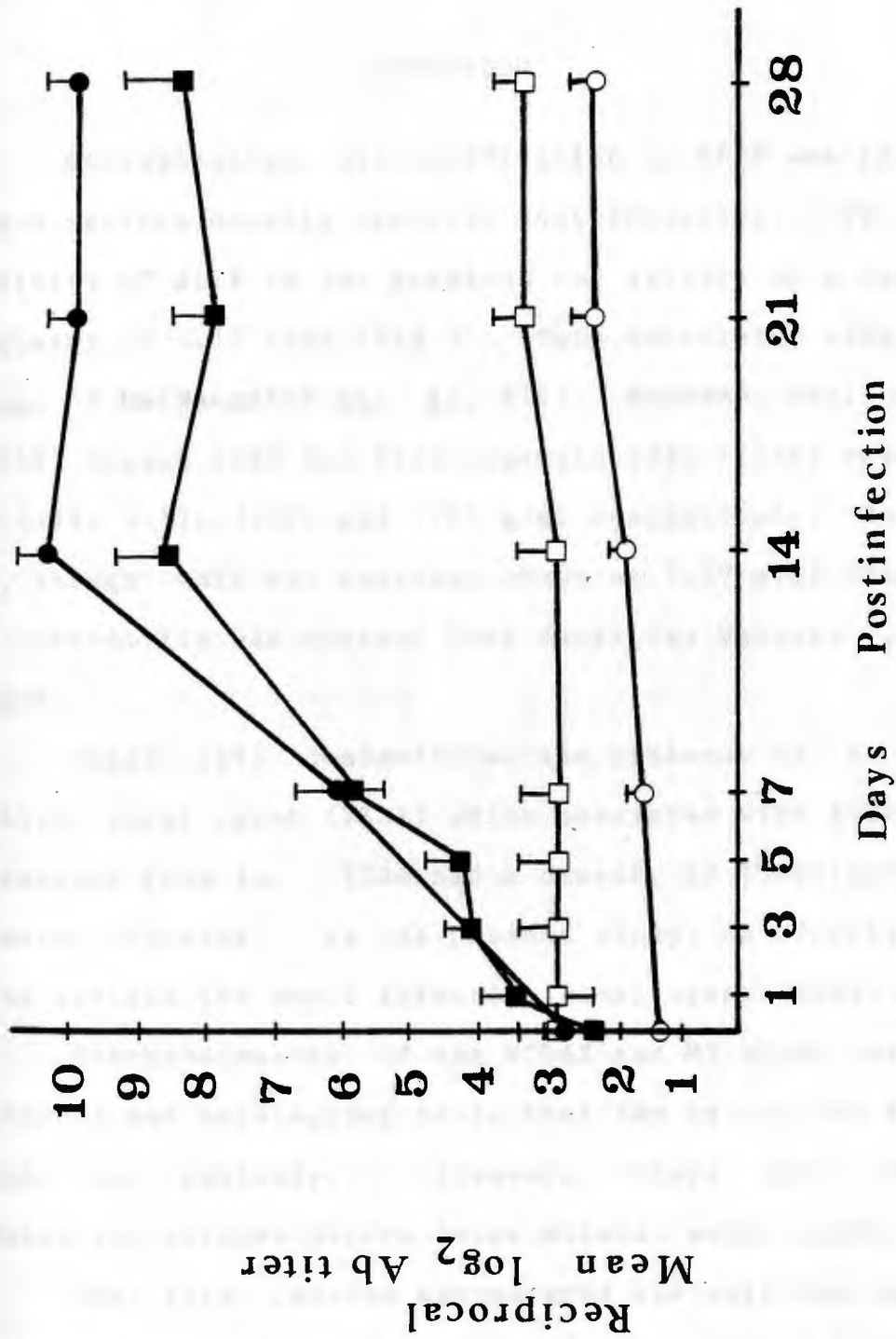
Table 9. Comparison of antibody response in noninfected and AIBV infected chickens^{b,c} from Experiment 1 and Experiment 2.

Days PI ^d		Reciprocal mean antibody titer $\log_2 \pm$ SE (N)	
		Experiment 1	Experiment 2
0	C ^e	2.6 \pm 0.40 (5) ^g	1.0 \pm 0 (7)
	I ^f	2.1 \pm 0.28 (10)	2.4 \pm 0.23 (12)** ^j
1	C	2.6 \pm 0.44 (5)	NT ^h
	I	3.2 \pm 0.42 (10)	NT
3	C	2.6 \pm 0.44 (5)	NT
	I	3.8 \pm 0.33 (10)* ⁱ	NT
5	C	2.6 \pm 0.44 (5)	NT
	I	4.0 \pm 0.45 (5)*	NT
7	C	2.6 \pm 0.40 (5)	1.3 \pm 0.18 (7)
	I	5.6 \pm 0.38 (9)*	5.8 \pm 0.43 (12)**
14	C	2.6 \pm 0.40 (5)	1.6 \pm 0.20 (7)
	I	8.2 \pm 0.73 (5)*	10 \pm 0.43 (12)**
21	C	3.0 \pm 0.45 (5)	2.0 \pm 0.25 (7)
	I	7.6 \pm 0.54 (10)*	9.6 \pm 0.43 (11)**
28	C	3.0 \pm 0.45 (5)	2.0 \pm 0.25 (7)
	I	8.0 \pm 0.72 (10)*	9.5 \pm 0.39 (11)**

- a. Antibody was detected by the modified indirect enzyme-linked immunosorbent assay
- b. Chickens were three-weeks of age
- c. Chickens were infected by intratracheal inoculation with 10^3 ELD₅₀/ml of AIBV
- d. [PI] = Postinfection
- e. [C] = Noninfected control chickens
- f. [I] = Infected chickens
- g. mean titer \pm standard error (total number of chickens)
- h. [NT] = Not tested
- i. [*] = $p \leq 0.02$
- j. [**] = $p \leq 0.001$

Figure 11. Immune response to infection with AIBV. Comparison of mean log₂ MI ELISA titers in Experiment 1 of control chicks [□] and chicks infected [■] intratracheally with 10³ ELD /ml of AIBV and in Experiment 2 of control chicks [○] and chicks [●] infected intratracheally with 10³ ELD /ml of AIBV. Bars represent the standard error (SE).





DISCUSSION

Concentration and purification of AIBV was carried out in a sucrose density gradient centrifugation. The peak infectivity of AIBV in the gradient was visible by a band at the density of 1.17 g/ml (Fig 1). This correlated with the findings of MacNaughton *et. al.* (16). However, Collins *et. al.* (15) showed AIBV had five separate infectivity peaks at 1.14, 1.16, 1.17, 1.19, and 1.21 g/ml respectively. In this study, though only one peak was shown at 1.17 g/ml (Fig 1), virus infectivity was present from densities between 1.13 to 1.23 g/ml.

Raggi (94) demonstrated the presence of a small infective viral agent (VIRA) which coexisted with AIBV but was distinct from it. VIRA had a density of 1.18-1.21 g/ml in cesium chloride. In the present study, no efforts were made to isolate the small infective viral agent (VIRA).

Standardization of the MIDAS and MI ELISA methods resulted in two serological tests that can be used to detect antigen and antibody. However, there were several obstacles to overcome before these methods were usable.

The first problem encountered was with the use of the microtiter plates. The plastics used in microtiter

plates display a negative electric potential at their surface (zeta potential) which is modified by adsorption of protein, but, not eliminated. This residual electrostatic field at the solid-solution boundary gives rise to a diffuse double-layer of ions at the interface. This double layer of ions containing free antigen or antibody can be displaced by vigorous or prolonged washing (76).

Polystyrene microplates are widely used as the plastic support in ELISA methods because they can be coated so easily and show reproducibility in results (80). Most proteins (ie. antibody and antigen) are absorbed to plastic surfaces as a result of hydrophobic interaction between non-polar protein substance and the non-polar plastic matrix and are not covalently bound (76). Unlike antigen-antibody interactions, this adsorption process is nonspecific.

However, this immunoadsorption process can cause a problem. Because of the physical attachment of protein, bleeding can occur causing a loss of adsorbed proteins during washing and incubation. This results in the loss of sensitivity due to non-specific binding of enzyme-labelled components which contribute significantly to the total bound enzyme activity. During incubation of the immobilized antigen or antibody with enzyme-labelled antibody or antigen, direct adsorption of the conjugate onto the solid plate can occur. This non-specific adsorption can be minimized by the inclusion of Tween-20, a neutral detergent

(76,77,80). This non-ionic detergent can be added in concentrations that prevent formation of new hydrophobic interaction between added protein and the solid phase. However, these detergents do not appreciably disrupt physical bonds already formed between previously adsorbed antigen or antibody and the plastic surface.

HRP was chosen as the enzyme conjugate because of its unusual stability, high reactivity, and easy storage (77). Use of this enzyme conjugated to the heavy and light chain anti-immunoglobulins allows the conjugate to react with the major heavy and light chain immunoglobulin determinates of all classes. Piela (92) stated that use of the HRP conjugated to the heavy and light chain anti-immunoglobulin, in the ELISA, only measured IgG. The investigator used this assumption to explain the difference observed between the ELISA and the HI test. This assumption was incorrect.

The substrate, OPDA, is colorless initially, but yields a colored product on degradation. OPDA is highly sensitive in the ELISA test, but, unfortunately, it is mutagenic and photosensitive (80).

The amount of conjugate added to the system is chosen by trial and error in order to give a satisfactory sensitivity (74). Sensitivity can be increased by lengthening the duration of the enzyme-substrate indicator reaction which results in the accumulation of the product with time.

A problem was encountered with the enzyme-substrate indicator system. Color development continued even after the addition of the stopping solution. This problem was reported before and could not be alleviated (77). This was one reason why the inclusion of positive and negative reference sera was important. Other reasons include changes in antigen or antigen concentration, dilution of antibody or conjugate or both which can effect the absorbance values. Inclusion of the reference samples help eliminate systematic components of error and gives analytical consistency to the absorbance value measurements (72,83).

In the MIDAS ELISA, problems of non-specific binding were overcome during optimization procedures. The final testing system had low non-specific absorbance values.

In the MI ELISA, the problems with non-specific binding of avian immunoglobulins as described by Slaughter et. al. (95) and the presence of inhibitors in the serum as described by Lukert (96) were generally not encountered. The addition of fetal calf serum to mask the polystyrene surface (89,97) was therefore not necessary.

Adsorption of AIBV onto the microtiter plate had the best result using a carbonate-bicarbonate buffer at an alkaline pH of 9.6. It was reported by Cunningham and Hofstad, that AIBV had maximum stability at pH 7.8 (1,7). However, Soula and Moreau (85) showed that the use of the carbonate-bicarbonate buffer at pH 9.6 had little effect on

the virus. The carbonate-bicarbonate buffer at pH 9.6 had no apparent effect on the virus in this study.

The prozone effect as described by Bruins *et. al.* (97) was encountered at low serum dilutions at the beginning of immunization. The early IgG antibodies were not able to resist washing procedures when crowded and also were not able to align on the antigen site at low serum dilutions. They did resist washing when they were not as crowded. During later immunization, the antibodies had a high affinity for the antigenic sites and were able to "hang-on" through washing even though crowded and poorly aligned.

In both the MIDAS and MI ELISA methods, unexplainable irregularities on the plate or the "plate effect" (73,80) was not generally encountered in this study.

In Experiments 1 and 2, the clinical and post-mortem results were similar to those previously reported (1,7).

In Experiment 1, virus was isolated from six out of six tracheal swabs of chickens for seven days. However, in Experiment 2 virus was isolated from six out of six tracheal swabs of chickens for fourteen days (Table 7). This difference could be attributed to the use of commercial chickens in Experiment 1 compared to the use of SPF chickens in Experiment 2.

In Experiment 1, the results of virus isolation by chicken embryos and the MIDAS ELISA showed comparable

sensitivity. However, dwarfing and death of chick embryos was not always evident, and as is shown in Table 1 several passages of the virus in chicken embryos may be necessary in order to isolate the virus in the test sample. The results of the MIDAS ELISA, on the other hand, does not need repeated passage of the test sample and can be photometrically analyzed, which eliminates the investigator's subjectivity.

In Experiment 2, the results of virus isolation by chicken embryos and the detection of viral antigen by the MIDAS ELISA differed slightly. There was a reduction in the isolation of AIBV in chicken embryos. This could be due to the presence of maternal antibody in the commercial eggs used for virus isolation (42). Another explanation for this difference is the MIDAS ELISA can detect and measure antibodies directed against both infectious and non-infectious viral antigens (87,89). In contrast, virus isolation in embryonated chicken eggs can only detect infectious viral antigens.

Results of the detection of viral antigen by the MIDAS ELISA demonstrated persistence of the viral antigen in the feces. This has also been reported to occur in a low proportion of chickens in other flocks (46). Studies assessing the duration of AIBV infections in chickens (46,47) show that AIBV may enter organs of the respiratory tract from the blood stream due to viremia. The virus can

also be present in the small and large intestines in great quantity for a relatively long period of time. Some investigators feel that AIBV can actually multiply in the intestines (46), although, this has not been proven to date. Regardless of this fact, AIBV can be detected in the feces of the chicken for extended periods of time and in the presence of humoral antibodies. How the virus accomplishes this feat is unknown.

Persistence of infection of chickens with AIBV is an important factor for the poultry industry to consider. Non-infected chickens sharing the same house with persistently infected chickens can become infected (46,47). Reinfection can also occur if there is a sufficient decrease in the chicken's protective antibodies. Stress can also induce clinical disease in those chickens persistently infected with AIBV (46).

The results of the detection of humoral antibody in infected chickens by the MI ELISA were comparable with results shown by other investigators using similar MI ELISA techniques and serum neutralization tests (78,79,87,88). This study showed that there was a loss in the detection of AIBV in the infected chickens as the humoral antibody titer of the chickens increased. This was probably due to coating of viral antigens by immune sera causing destruction of the antigen by the avian immune system (12, 98).

It has been suggested by several investigators that

virus isolation attempts should be done within ninety-six hours postinfection (99,100). Results in this study tend to agree with this recommendation. This time restriction should also apply to the MIDAS ELISA.

The ELISA methods are an important means of detecting infectious agents and immune responses in animals. Even with the countless possible permutations of variables used in the ELISA methods, the ease of procedure, low cost of materials, reagent stability, short test time, safety, sensitivity, reproducibility, are many advantages of the ELISA methods (75,76,77,78,79,80,81,82,84,85,86,87,88,89,90,91,92,93,97).

There is however a need for an ELISA that is uniform and can be applied universally. Presently, it is difficult to compare inter-laboratory results. Since there is no standard ELISA plan, intra-laboratory results can only be compared by use of a positive and negative reference serum or antigen. The value of the MIDAS ELISA for detecting antigen and the value of the MI ELISA for detecting antibodies can only be ascertained if all laboratories pool their resources together to create a universal system.

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Table 1. Mortality and morbidity data for 1971-72

	a	b	c
Infective dose (ID ₅₀)	100	100	100
Number affected	5	2	1
Number surviving	1	2	1
Approximate the affected (Total starting at each dilute level) approximate and approximate level	100	100	100
Approximate the unaffected (Total starting at each concentrate level)	100	100	100
Express the mortality rate (No. affected/total)	5%	2%	1%
Express % mortality	50%	20%	10%

a = total concentrated dilution data series (100% ID₅₀)
 b = the first dilution below 50% ID₅₀
 c = the first dilution below 10% ID₅₀
 d = the number of animals affected/total and surviving

To compare the mortality and morbidity data for 1971-72

For each mortality rate - For each mortality rate
 above 50% - above 50%
 a proportional distance

Find the log of each mortality rate and the mortality rate
 above 50% - mortality rate proportional distance 2 log
 of the dilution factor - to obtain the log value of the
 proportional distance (2 log) to the nearest decimal.

APPENDIX A

Determination of embryo lethal dose fifty percent in embryonated chickens eggs.

Dilutions of Virus Spanning the 100 % to 0 % Affected Range			
	a	b	c
	10	10	10
Infective (death) rate	4/4	2/4	0/4 ^d
Number affected	4	2	0
Number surviving	0	2	4
Accumulate the affected (Total starting at most dilute level; accumulate most concentrate level)	= 4 +	= 2 +	
6		2	0
Accumulate unaffected (Total starting at most concentrate level; accumulate towards most dilute level)	= 2 +	= 4 +	
0		2	6
Express the mortality rate (No. affected/total)	6/6	2/4	0/6
Express % mortality	100 %	50 %	0 %

- a = most concentrated dilution that shows 100 % affected
- b = the next dilutions between "a" and "b"
- c = most dilute level that shows 0 % affected
- d = the number of embryos affected/total per dilution tested

To compute the actual end point dilution use the following formula:

$$\frac{\text{per cent mortality next above 50 \%} - 50 \%}{\text{Per cent mortality next above 50 \%} - \text{Per cent mortality next below 50 \%}} = \text{proportionate distance.}$$

Find the log of that dilution in which per cent mortality is next about 50 %. Multiply the proportionate distance X log of the dilution factor 10 to obtain the real value of the proportionate distance (round to the nearest tenth).

APPENDIX B

Plate I. AIBV from infected AAF of 10-day-old embryonated chicken eggs was harvested after 30 hours PI and clarified at 7,650 x g for 10 minutes. The virus was then stained using pseudoreplication. The stained grids were viewed with a Hitachi HS-9 EM at 75 Kv. The corona are indicted by an arrow. (Mag 269,000 X)



Plate II.

The ELISA antigen-antibody complex. AIBV from infected AAF of 10-day-old embryonated SPF eggs was harvested after 36 hours PI and clarified at 7,650 x g for 10 min. The virus suspension was then pelleted at 76,700 x g for 1 1/2 hours. The pellet was resuspended in PBS. Chicken anti-AIBV was added to the virus suspension and incubated for 30 min at 37 °C. The virus-antibody mixture was then stained using the agar block method. The stained grid was view with a Hitachi HS-9 EM at 75 Kv. Corona are indicated by arrows. (Mag 286,000 X)

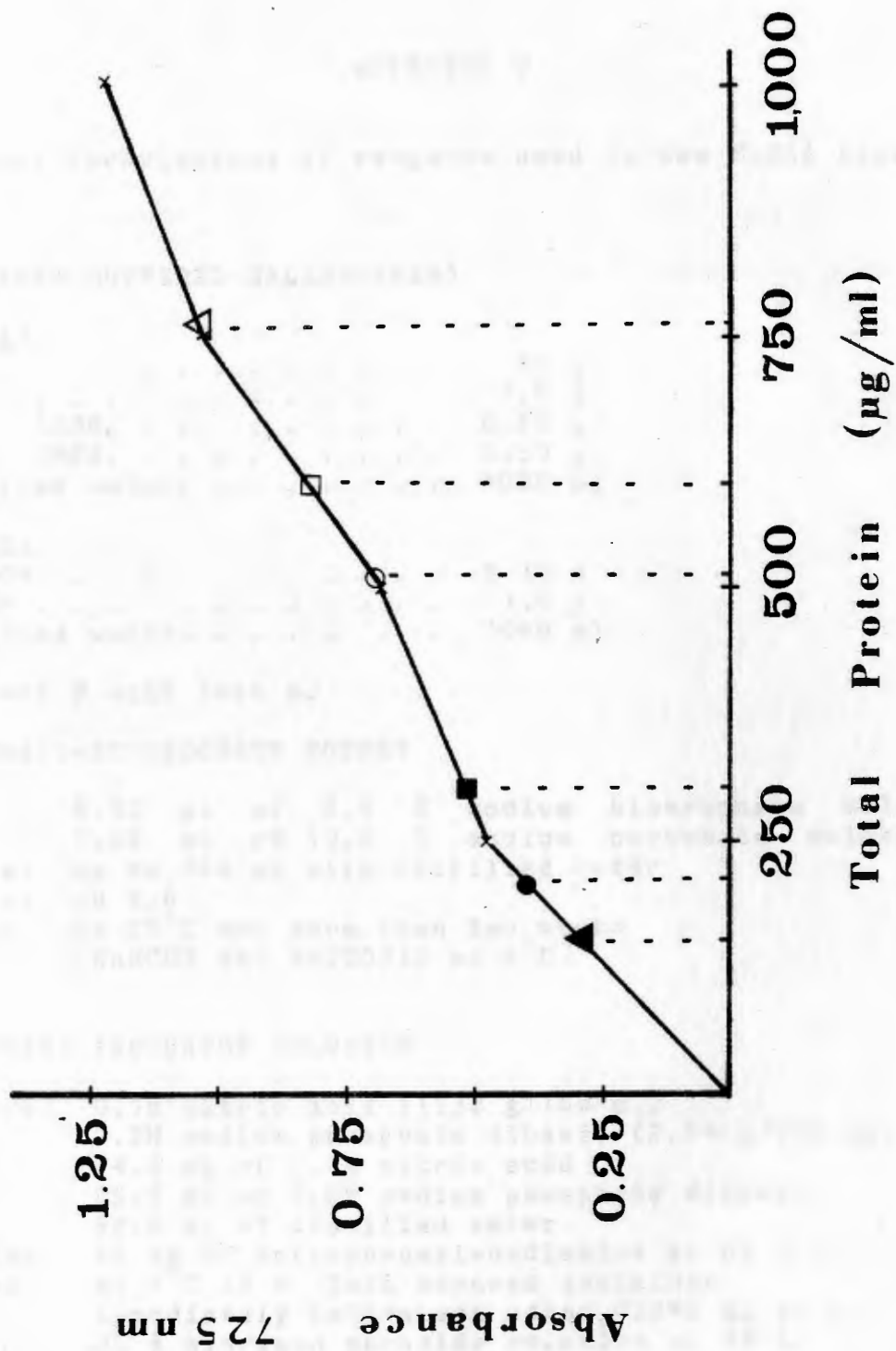


APPENDIX C

Determination of protein concentrations of various ELISA test reagent was carried out. In Fig. 12, protein concentration vs absorbance (725 nm) was plotted to obtain a calibration curve. Various test samples' absorbance values were then extrapolated and their protein concentrations determined.

Figure 12.

Protein concentrations of antigen and antibody samples were plotted on a calibration curve which used bovine albumin as a protein standard. The known protein concentrations (x) were read at an absorbance value of 725 nm and plotted. The test samples: chicken anti-AIBV (CAIBV)[○], rabbit anti-chicken IgG conjugated to HRP (RAC-HRP) [●], rabbit anti-AIBV (RAIBV)[□], goat anti-rabbit IgG conjugated to HRP (GAR-HRP) [■], ELISA antigen (ELISA Ag) [△], and purified AIBV (P-IBV) [▲]; absorbance values were read and extrapolated on the calibration curve to determine the protein concentration (mcg/ml).



APPENDIX D

Chemical formulations of reagents used in the ELISA test.

PHOSPHATE BUFFERED SALINE (PBS)

Part A:

NaCl	40 g
KCl	1.0 g
MgCl ₂ 6H ₂ O	0.66 g
CaCl ₂ 2H ₂ O	0.50 g
Distilled water	4000 ml

Part B:

Na ₂ HPO ₄	5.75 g
KH ₂ PO ₄	1.0 g
Distilled water	1000 ml

Mix Part B with Part A.

CARBONATE-BICARBONATE BUFFER

Add: 4.53 ml of 8.4 % sodium bicarbonate solution
1.82 ml of 10.6 % sodium carbonate solution
Dilute: up to 100 ml with distilled water
Adjust: pH 9.6
Store: at 25°C not more than two weeks
[NaHCO₃ and Na(CO₃)₂ at 4°C]

SUBSTRATE INDICATOR SOLUTION

Prepare: 0.1M citric acid (1.92 g/100 ml)
0.2M sodium phosphate dibasic (2.84 g/100 ml)
Add: 24.3 ml of 0.1M citric acid
25.7 ml of 0.2M sodium phosphate dibasic
50.0 ml of distilled water
Mix in: 40 mg of orthophenylenediamine at pH 5.0
Store: at 4°C in a foil covered container
Use: immediately before use added 0.040 ml of a
30 % hydrogen peroxide solution at 25°C.

MIDAS ELISA

APPENDIX E

Diagrammatic illustration of steps involved in the modified indirect double antibody sandwich (MIDAS) enzyme-linked immunosorbent assay (ELISA).

1. 1^o Ab step
[CAIBV]
Y



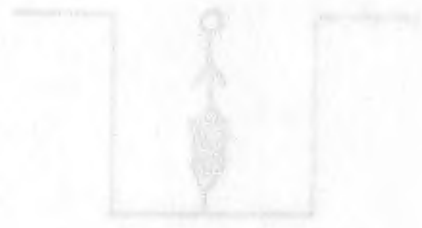
2. Ag sample step
[CAIBV]
●



3. 2^o Ab step
[RAIBV]
λ



4. Conjugate step
[GAR IgG-HRP]
λ

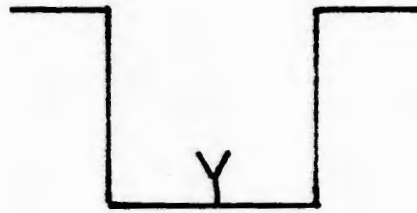


5. Substrate step
[OPDA]
* * *



MIDAS ELISA

1. 1^o Ab step
[CAIBV]
Y



2. Ag sample step
[AIBV]
◆



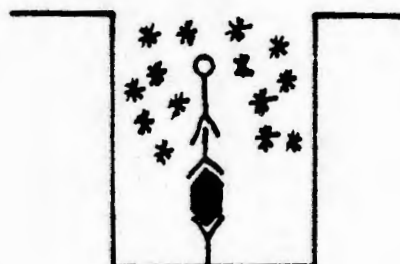
3. 2^o Ab step
[RAIBV]
∧



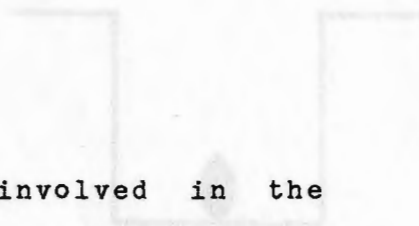
4. Conjugate step
[GAR IgG-HRP]
∧



5. Substrate step
[OPDA]
* * *



MI ELISA

1. Ag step
(AIBV) APPENDIX F

Diagrammatic illustration of the steps involved in the modified indirect (MI) enzyme-linked immunosorbent assay (ELISA).

2. Ab step
(CAIBV)3. Conjugate step
(RAC IgG - HRP)4. Substrate step
(O P D A)

MI ELISA

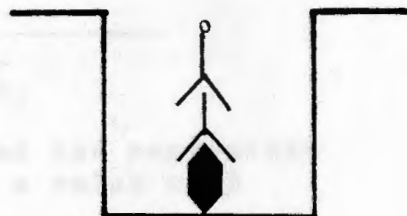
1. Ag step
(AIBV)



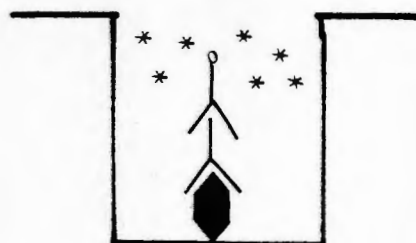
2. Ab step
(CAIBV)



3. Conjugate step
(RAC IgG - HRP)



4. Substrate step
(OPDA)



APPENDIX G

Statistical Methods and Formulas

Mean: $\bar{x} = 1/n \sum x$

where n is the number of observation in samples
and x is the observed measurements

Standard deviation: $s = \sqrt{1/n-1 \sum (x-\bar{x})^2}$;

$$\sum (x-\bar{x})^2 = \sum x^2 - 1/n (\sum x)^2$$

Variance: s^2

Standard error (SE): $\bar{x} \pm s/\sqrt{n}$

Comparison of means of two small samples (unknown variances assumed to be equal):

$$s^2 = \frac{\sum_1 (x_1 - \bar{x})^2 + \sum_2 (x_2 - \bar{x})^2}{n_1 + n_2 - 2}$$

$$t = \frac{\bar{x}_1 - \bar{x}_2}{s \sqrt{1/n_1 + 1/n_2}}$$

use one-tailed Student's t-test to find the percentage point for the probability of observing a value of t

Confidence limits: $\bar{x} \pm ts/\sqrt{n}$ (P = 0.05)

ABBREVIATIONS

AE = Avian encephalomyelitis
AGP = agar gel precipitation
AIB = Avian infectious bronchitis
AIBV = Avian infectious bronchitis virus
C = control group of chickens
CAIBV = chicken anti-avian infectious bronchitis virus
CAM = chorioallantoic membrant
CAS = chorioallantoic sac
CEKC = chicken embryo kidney cells
CF = compliment-fixation test
ELD₅₀ = median embryo lethal dose
ELISA = enzyme-linked immunosorbent assay
EM = electron microscope
F = fecal swabs
GAR IgG = goat anti-rabbit immunoglobulin G
HI = hemagglutination inhibition
HRP = horseradish peroxidase
I = infected group of chickens
IF = immunofluorescence
ILT = infectious laryngotracheitis
Kv = Kilovolts
M-41 = Massachusetts strain of AIBV
M-42 = Beaudette strain of AIBV
M-46 = Connecticut strain of AIBV
MI = Modified indirect
MIDAS = Modified indirect double antibody sandwich
NANA = N-acetylneuraminic acid
NDV = Newcastle disease virus
NT = not tested
OPDA = orthophenylene diamine
PBS = Phosphate buffer saline
PI = postinfection
PSF = penicillin, streptomycin, and fungizone
PTA = phosphotungstic acid
RAC IgG = rabbit anti-chicken immunoglobulin G
RAIBV = rabbit anti-AIBV
RI = reinoculation
RNA = ribonucleic acid
SPF = specific pathogen free
T = tracheal swabs
VI = virus isolation
VN = virus neutralization
v = volume
w = weight

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