SPECTROPHOTOMETRIC AND SERUM NEUTRALIZATION STUDIES OF SERA FROM CHICKENS EXPOSED TO INFECTIOUS BRONCHITIS VIRUS

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY

Osmano Hipólito

1955

THESIS

This is to certify that the

thesis entitled

Spectrophotometric and Serum Neutralization Studies of Sera from Chickens Exposed to Infectious Bronchitis Virus

presented by

Osmane Hipolito

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Microbiology and Public Health

Major professor

Date August 26, 1955

O-169

SPECTROPHOTOMETRIC AND SERUM NEUTRALIZATION STUDIES OF SERA FROM CHICKENS EXPOSED TO INFECTIOUS BRONCHITIS VIRUS

By

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A THESIS

Submitted to the School of Advanced Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1955

ABSTRACT

Infectious bronchitis of chickens (IB) is an acute and highly contagious disease of great economic significance to the poultry industry.

The purpose of the present investigation was to study the relationship of spectrophotometric analyses of serum as compared to antibody response measured by the serum neutralization test following inoculation of susceptible birds with infectious bronchitis virus.

Adult, single-comb, White Leghorn cockerels were used as experimental chickens.

Two strains of virus were used. Strain V114D, an egg-adapted strain, was employed in all the serum neutralization tests. Strain VL was isolated from field cases of the disease and was used to infect susceptible birds.

Chickens were bled by intracardiac puncture immediately prior to exposure to IBV and then after three, five, seven, ten, twelve, sixteen, and twenty weeks. Serum samples were stored at -30°C until used.

Sera of individual birds were analyzed with the Beckman Model B spectrophotometer before and after chemical fractionation by the

method of Wolfson et al. The following values were determined: total protein, albumin, and alpha, beta, and gamma globulins.

Sera from individual birds bled at the same time interval were pooled in equal portions and analyzed spectrophotometrically. Serum neutralization tests and macro-Kjeldahl determinations were also performed on these pools.

A close agreement was observed in the total protein determinations by the Kjeldahl and the spectrophotometric methods although the values obtained with the former method were slightly higher.

Results obtained by spectrophotometry after chemical fractionation showed a fair agreement with those found by several investigators with the use of the electrophoresis apparatus.

There was a close agreement of the results obtained with sera of individual chickens and those of pooled sera with respect to total protein, albumin, total globulin, gamma globulin, and A/G ratios. Alpha and beta globulin results showed a marked variation.

A decrease in albumin values and consequently an inversion of the A/G ratios was observed after IBV inoculation. Alpha and beta globulins were specifically responsible for the increase in globulins. Gamma globulin had no influence on this change since its values were almost uniform during the course of the investigation.

In an endeavor to explain the results obtained it must be emphasized that a large amount of blood (20 ml., or approximately one-fourth of the total blood volume) was removed from each bird at every bleeding period. Although the intervals between bleedings were never less than two weeks, it is possible that this interval might not have been sufficient for the complete restoration of the original balance of the serum components.

Serum neutralization tests conducted on pooled sera showed typical response with infection by IBV. A maximum titer in antibodies was found between the seventh and twelfth weeks after inoculation.

There was no correlation between the changes in serum components and antibody content. When the serum components had returned to preinfection levels the antibody content was at the maximum.

To

MY FAMILY

this manuscript is

most affectionately dedicated

ACKNOWLEDGMENTS

The author is grateful for the generous help, guidance, and instruction given to him by Dr. Charles H. Cunningham, Professor of Microbiology and Public Health. He is also indebted to Dr. Erwin J. Benne and the other staff members of the Department of Agricultural Chemistry, responsible for the protein nitrogen analyses. Grateful acknowledgment is also due to Mrs. Martha P. Spring.

The writer deeply appreciates the financial support of the Universidade Rural do Estado de Minas Gerais (Brazil) and of The Rockefeller Foundation, which made it possible for him to complete this investigation.

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Infectious bronchitis of chickens is a disease of great economic significance to the poultry industry.

The purpose of the present investigation was to study the relationship of spectrophotometric analyses of serum as compared to antibody response measured by the serum neutralization test following inoculation of susceptible chickens with infectious bronchitis virus.

REVIEW OF LITERATURE

Infectious Bronchitis of Chickens

Infectious bronchitis (IB) is an acute and highly contagious respiratory disease of chickens first reported by Schalk and Hawn (85) in North Dakota in 1931. The infection has subsequently been observed throughout the United States (3, 5, 8, 9, 17) and in England (1, 5), Canada (5), and Holland (87).

The disease is apparently specific for chickens and affects all ages, breeds, and sexes. The morbidity is high but the mortality is variable. In chicks the mortality may be as high as 90 per cent, but in birds over six weeks old the loss is generally negligible. Infectious bronchitis constitutes a serious economic problem in view of its rapid spread and the marked decrease in egg production of affected laying flocks (34, 88).

Etiology

Infectious bronchitis is caused by a virus, Tarpeia pulli. This species designation was suggested by Packer (73) in 1950.

Infectious bronchitis virus (IBV) is spherical, ranging from 65 to 135 millimicrons with an average of 70 millimicrons (80, 81).

The virus may be passed through all grades of Seitz (34) and Berkefeld filters (6, 10, 34).

The etiologic agent can be isolated from the lung and trachea throughout the respiratory phase of the disease (43).

Transmission

Transmission is either by contact or aerosol with a rapid spread from infected to noninfected chickens.

Experimentally the disease can be readily transmitted by intratracheal and intranasal inoculations (10, 34), but not by subcutaneous or intramuscular inoculation.

The virus can be transmitted throughout the respiratory stage of the disease and has been recovered from infected chickens for as long as four weeks after inoculation (45).

Carriers may be capable of transmitting the virus as long as forty-three days after an outbreak (65), and in some instances as long as two months (34).

Hofstad (52) was unable to demonstrate the existence of carriers of IB among recovered birds by using the direct contact method.

Symptoms

Symptoms usually appear twenty-four hours after intratracheal inoculation, but in some cases it may be several days before they can be observed. The majority of affected birds manifest symptoms from one to eleven days after inoculation.

Severity of the infection varies with the virulence of the virus, conditions of the flock, and individual susceptibility.

Gasping, coughing, sneezing, and tracheal rales are the most prominent symptoms (6, 8, 33, 53, 85). Fluidlike feces appear in some cases (88).

The most important aspect of the disease in laying flocks is the marked decline in egg production which may persist for many weeks. Eggs produced by hens recovered from the disease are misshapen, rough, soft-shelled, and of poor quality (16, 70, 89). In many instances several months may elapse before a flock returns to the preinfection level of egg production. Many hens suffer permanent ovarian damage.

Lesions

Lesions are generally confined to the lungs and bronchi (17, 85). These alterations consist of pulmonary congestion and a serous

or catarrhal exudate in the trachea. Pleuritis and pericarditis may also be observed.

In young chicks pathologic alterations are variable, but edema of the mucosa of the trachea and bronchi and a serous or purulent exudate are uniformly observed. Coagulation of this exudate forms plugs that occlude the lumen of the larynx and produce death by asphyxia. Other lesions are an edematous thickening of the tracheal mucosa and submucosa and diffuse, leucocytic infiltration with little desquamation and complete absence of gross hemorrhage. Neither inclusion bodies nor pathognomonic lesions were found in experimental and field cases of the disease (51).

In semimature chickens tracheitis is the most constant lesion. In mature birds the tracheal reaction may be more severe and extensive (88). A noncellular film may be found by microscopic examination of the tracheal mucosa of some birds (51).

Lesions in the nervous system have not been observed (8).

Resistance of Virus to Physical and Chemical Agents

Tracheal exudate when frozen, dried, and stored at 4°C. remains infective for 180 days. The virus retains its virulence for eighty days when stored in 50 per cent glycerin at 4°C. (6). At room temperature virus-infected allantoic fluid remains viable for

five to seven days, but not for fourteen days. At 50°C. the virus is viable for fifteen minutes (35).

Freezing and thawing has no harmful effect on the virus. A hundred fold decrease of infective doses occurs in lyophilized samples after seven days' storage at 4°C. This decrease may be due to incomplete drying of the virus preparation (28).

The virus is more stable at 4°C. in an acid medium than in an alkaline medium for sixty days. The reverse is observed from sixty to 170 days with an optimum of pH 7.80 (27).

Inactivation of the virus is achieved in three minutes or less by 1 per cent phenol, 1 per cent liquor cresolis saponatus, 1 per cent metaphen, 1:10,000 $KMnO_4$, 1:1,000 $HgCl_2$, 95, 70, 40, and 25 per cent ethanol, 1:1,000 tincture of Zephiran, 1 per cent Lugol's solution, 1:20 NaOH, 5 per cent Neoprontosil, and 1 per cent formalin (26).

Diagnosis

Clinical symptoms and history may be employed only for a presumptive diagnosis.

Definitive identification of infectious bronchitis can be accomplished in the laboratory by: (1) isolation of the virus in embryonating

chicken eggs, or (2) serum neutralization tests for the identification of specific antibodies (6, 10, 34).

Isolation of the virus

Cultivation of IBV in embryonating chicken eggs was first reported in 1937 by Beaudette and Hudson (10), who used the chorioallantoic membrane route of inoculation.

Beaudette and Hudson (10) and Delaplane and Stuart (35) found that the first few passages of the virus in embryonating chicken eggs by the chorioallantoic membrane route there was but slight embryo mortality. With successive passages, virus increased in virulence for the embryo. With some strains complete adaptation of the virus to the embryo with respect to the mortality occurred by the sixty-fifth passage. With other strains this was observed at the ninetieth and one hundred twenty-fifth passages. Completely egg-adapted strains produced 100 per cent mortality on the second day after inoculation.

Delaplane and Stuart (35) reported later that IBV adapted earlier to embryonating chicken eggs by inoculation via the allantoic cavity rather than the chorioallantoic membrane.

IBV can be readily isolated and propagated in embryos by the chorioallantoic and amnionic and allantoic cavity routes of inoculation (29, 59).

Increased virulence of the virus for embryo by successive passages is accompanied by a decrease in pathogenicity for chickens. Completely egg-adapted virus is nonpathogenic and nonantigenic for chickens (53).

Cunningham and El Dardiry (25) studied the distribution of the virus in embryonating chicken eggs following inoculation via the allantois and found a higher concentration in the chorioallantoic membrane followed in decreasing order by allantoic fluid, amnionic fluid, and liver. Yolk material was innocuous. A decrease in titer was observed when eggs were left in the incubator after death of the embryo. An interfering substance in the allantoic fluid of such eggs was detected by Groupé (49).

Curling and dwarfing of the embryo are the outstanding gross alterations following inoculation with virus initially isolated from chickens (10, 34, 42, 70). These lesions are pathognomonic of infection with IBV (70). Thinning and adherence of the chorioallantoic membrane to the shell membrane, fibrosis of the amnion, sluggish and weak embryos, and immature feather development are also observed. Microscopic alterations include proliferation of mesodermal and ectodermal cells, edema of the amnionic membrane, necrosis and hemorrhage of the liver, interstitial nephritis, congestion of

the spleen, and slight capillary congestion of the brain. Inclusion bodies are not found.

Serum neutralization test (SN)

Ability of anti-infectious bronchitis serum to neutralize IBV was first reported by Beach and Schalm (6). Mixtures of virus and serum were not infectious for susceptible chickens. This method was later used with embryonating chicken eggs.

It was found by Cunningham (22) that the serum neutralization titers of normal birds not previously exposed to IBV would not be expected to exceed $10^{1.517} \pm 10^{0.0376}$, or thirty-six neutralizing doses. Fabricant (44) suggested a minimum of one hundred neutralizing doses for the diagnosis of the disease. This level is usually obtained in serum collected from chickens three weeks after infection (79).

Jungherr and Terrell (60) and Fabricant (44) found that the SN test is reliable when the virus containing at least 10^4 to 10^6 embryo infective doses is used and if certain technical details are observed.

According to Page (79), the results obtained with the serum neutralization test employing serum diluted 10^{-1} and 10^{-2} may be adjusted with the respective log unit to give an accurate assessment

of the results that would have been obtained by using undiluted serum.

IB-immune serum undergoes no significant change in neutralizing capacity following exposure to 4°C for eight weeks, seven days at 22°-25°C., and at 37°C. a tenfold decrease occurs after fifty-six hours (79).

The LD_{50}NI of pooled sera varied approximately 0.4 to 1.0 log unit from the average LD_{50}NI of the individual serum samples comprising the pooled samples. No explanation was offered (38).

IBV fails to agglutinate chicken red blood cells and the hemagglutination-inhibition test cannot be used to identify either the virus or specific antibodies (4, 24, 30, 32, 41).

Control

Birds recovered from infectious bronchitis are immune for at least one year, but in some cases an overwhelming infection may reestablish the disease (69).

Naturally acquired passive immunity in chicks was first described by Jungherr and Terrel (60). Hofstad and Kenzy (54) have shown that five-day-old chicks with high SN titers were susceptible to the disease.

Healthy young birds can be successfully immunized by exposure to chicken-propagated IBV with little or no retardation in growth (7, 30, 34, 68).

Immunization of commercial flocks was first based upon this principle. Young birds preferably between ten to fourteen weeks of age were inoculated intratracheally with chicken-propagated IBV. About 1 per cent of the flock was inoculated and the disease spread to the rest of the flock in a few days (31, 88). This program had limitations such as the lack of a standardized virus, the possibility of transmission to susceptible flocks, the failure of "takes" in some birds, and the appearance of concomitant infections (88).

Commercially available live-virus infectious bronchitis vaccines contain virus cultivated in embryonating chicken eggs through a sufficient number of passages to decrease the virulence of the virus but to retain sufficient immunogenicity. These vaccines may be administered intranasally to individual birds, incorporated in the drinking water for autoinoculation, or applied as a dust for inhalation inoculation.

MATERIAL AND METHODS

Experimental Animals

Eleven adult, single-comb, White Leghorn cockerels, obtained from the United States Regional Poultry Research Laboratory, East Lansing, Michigan, were used in the experiment.

These birds were maintained in batteries in previously disinfected quarantine quarters.

A commercial growing mash[1] containing not less than 18 per cent protein, not less than 3.50 per cent fat, and not more than 6.50 per cent fiber was fed ad libidum.

Virus Antigens

Two different strains of IBV were used.

Strain V114D, embryo-adapted IBV, capable of killing all embryos inoculated via the allantoic cavity in twenty-four hours was used as the antigen in all in vitro serum neutralization tests. This strain was originally isolated by Beaudette and has been maintained in this laboratory for innumerable passages in embryonating chicken eggs.

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[1] Michigan State Growing Mash, manufactured by A. K. Zinn & Co., Battle Creek, Michigan.

Strain VL, chicken-propagated IBV, in the form of infected lung and trachea, was used for inoculation of all experimental chickens.

Within one week prior to initiation of the experiment, inoculum was prepared by grinding the lung and tracheal material with sterile sand and saline with a mortar and pestle. The suspension was passed through sterile cotton and cheese cloth and the filtrate was centrifuged at low speed to further separate the tissue particles. Approximately 0.2 ml. of the supernatant fluid was used to infect four six-week-old chickens by intranasal and intratracheal instillation. The tracheas were scraped with sterile cotton swabs to facilitate more intimate contact of the virus with the tracheal epithelium.

On the second day after inoculation typical symptoms were observed. The birds were killed and lungs and tracheas harvested and stored at -30°C. This material was then prepared as previously described and was used for inoculation of the experimental chickens.

For further identification of strain VL, five nine-day-old embryonating chicken eggs were inoculated with the virus via the allantoic cavity. Characteristic dwarfing and curling of the embryos was observed. Allantoic fluid harvested from the embryos failed to show hemagglutination activity.

Blood samples for SN tests and spectrophotometric analyses were obtained immediately prior to infection of the experimental chickens and at three, five, seven, ten, twelve, sixteen, and twenty weeks' postinoculation intervals. The chickens were fasted for eighteen to twenty-four hours before each bleeding to decrease the amount of lipids in the serum. Intracardiac puncture was employed by using sterile 20 ml. syringes fitted with 18 gauge, two-inch needles. Between bleedings the syringe and needles were washed with sterile saline.

About 20 ml. of blood was collected from each bird, transferred to a large test tube and allowed to coagulate in an inclined position. The coagulum was loosened by a sterile applicator stick and allowed to stand for twelve to sixteen hours at room temperature. Approximately 7 to 10 ml. of serum was obtained from each sample. The serum was centrifuged at 2,500 r.p.m. for ten to fifteen minutes, transferred to a sterile screwcap vial, and stored at -30°C. to prevent any deleterious changes prior to use. In some instances this period was as long as seven to eight weeks.

Since the amount of blood collected was approximately one-fourth of the total amount of blood in a chicken, bleeding intervals of not less than two weeks were employed to prevent protein depletion. At the end of twenty weeks only two out of eleven chickens

were alive. Fatality of the others was attributed to collection of blood and perhaps to severity of the disease.

Serum Studies

All individual sera were analyzed with the spectrophotometer before and after chemical fractionation. The following values were determined: total protein, albumin, and alpha, beta, and gamma globulins.

In addition, sera collected at the same time interval were pooled in equal portions and analyzed as described above. Serum neutralization tests and macro-Kjeldahl[1] determinations were also performed on the pools.

Chemical Fractionation

The procedure of Wolfson et al. (91) was employed as outlined below:

Reagents

1. Twenty-three per cent sodium sulfate solution: Dissolve exactly 23.0 grams of anhydrous sodium sulfate in distilled water at

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[1] Wilfarth-Gunning modification of the Kjeldahl method.

37°C. Make up to 100 ml. with distilled water and store in an incubator at 37°C.

2. Twenty-eight per cent sodium sulfite solution: Dissolve exactly 28.0 grams of anhydrous sodium sulfite in distilled water at 28°C. It is difficult to make the salt soluble, but it can be dissolved with sufficient shaking. Make up to 100 ml. with distilled water and store at room temperature.

3. Saline ammonium sulfate solution: In 1 liter volumetric flask, dissolve 193 grams of ammonium sulfate in about 500 ml. of distilled water. Add 40 grams of sodium chloride, dissolve and make up to 1 liter with distilled water. Store at room temperature.

4. Biuret reagent, Weichselbaum (90): Prepare an accurately titrated 0.2N NaOH solution. Dissolve 90 grams of Rochelle salt in about 400 ml. of this solution. Following solution, add 10 grams of $CuSO_4 \cdot 5H_2O$. When the copper sulfate has entirely dissolved add 10 grams of potassium iodide and make up to 2 liters with 0.2N NaOH. Store in a rubber-stoppered waxed glass bottle.

5. Span-ether reagent: Mix 1 ml. of Span 20 (Atlas Powder Co., Wilmington, Delaware) with 99 ml. of ether, U.S.P. Filter through a moderately fast paper into a 100 ml. graduated cylinder and make up to 100 ml. with ether. Store in a tightly corked bottle.

6. Ether, U.S.P.

Determinations

1. Total protein:

   (a) Pipette 0.2 ml. of serum into a 10 ml. graduated cylinder and dilute to 5 ml. with distilled water. Mix well by inversion.

   (b) Transfer 3.0 ml. to a cuvet, add 3.0 ml. of biuret reagent and mix well by shaking.

   (c) Prepare the blank with 3.0 ml. of distilled water and 3.0 ml. of biuret. Save this blank for use in the determination of albumin plus alpha globulin (Step 2f) and in the determination of gamma globulin (Step 8g).

   (d) Let the solutions stand at least 30 minutes. (Since the color is quite stable, readings may be deferred for as much as 24 hours.) Read on the photoelectric colorimeter or spectrophotometer at a wavelength of 540 millimicrons.

2. Albumin plus alpha globulin:

   (a) Place 2.3 ml. of 23.0 per cent sodium sulfate solution in a test tube. This solution is best not pipetted since it has a definite tendency to crystallize, particularly on cold pipettes. We have found it convenient

to use small calibrated tubes marked at 2.3 ml. and to fill these rapidly from a large burette, preparing enough at one time for a day's determination.

(b) Pipette in 0.2 ml. of serum and mix thoroughly by inversion.

(c) Add approximately 1.0 ml. of ether and shake vigourously for 30 seconds. Centrifuge for 5 to 10 minutes at 1,500 to 2,000 r.p.m.

(d) After centrifugation, carefully insert a pipette through the ether layer and beneath the packed globulin, slanting the tube to separate the precipitate from the wall of the tube.

(e) Withdraw 1.5 ml. of clear supernatant fluid and transfer to a cuvet. Add 1.5 ml. of distilled water and 3.0 of biuret reagent. Mix well by shaking.

(f) The blank is that prepared in Step 1c.

(g) After standing at least 30 minutes, read on the photoelectric colorimeter at a wavelength of 540 millimicrons.

3. Albumin:

(a) Place 4.8 ml. of 28.0 per cent sodium sulfite solution in a test tube. It is suggested that a procedure

similar to that advised in Step 2a be used in preparing these tubes.

(b) Pipette 0.2 ml. of serum and mix thoroughly by inversion.

(c) Add about 1.0 ml. of Span-ether reagent and invert gently 5 to 10 times. Centrifuge for 5 to 10 minutes at 1,500 to 2,000 r.p.m.

(d) After centrifugation, carefully insert a pipette through the Span-ether layer and beneath the packed globulin, slanting the tube to separate the precipitate from the wall.

(e) Withdraw 3.0 ml. of the clear supernatant fluid and transfer to a cuvet. Add 3.0 ml. of biuret reagent and mix well by shaking.

(f) Prepare the blank with 3.0 ml. of sodium sulfite solution and 3.0 ml. of biuret reagent.

(g) After standing for at least 30 minutes, read on the photoelectric colorimeter at a wavelength of 540 millimicrons.

4. Total globulin: Subtract the value for albumin obtained in Step 3g from the value for total protein obtained in Step 1d.

5. True albumin/globulin (A/G) ratio: Divide the value for albumin obtained in Step 3g by the value for total globulin obtained in Step 4.

6. Alpha globulin: Subtract the value for albumin obtained in Step 3g from the value for albumin plus alpha globulin obtained in Step 2g.

7. Beta globulin plus gamma globulin: Subtract the value for albumin plus alpha globulin obtained in Step 2g from the value for total protein obtained in Step 1d.

8. Gamma globulin:

(a) Pipette 9.6 ml. of saline ammonium sulfate into a sturdy 15 ml. thick-walled glass or plastic test tube. Layer 0.4 ml. of serum on top of the saline ammonium sulfate. Mix the two components by careful, slow, repeated inversion. Continue mixing until, within a minute or two, the gradually developing visible turbidity has reached an apparent maximum.

(b) Remove 1.0 ml. of the mixture with a pipette and discard.

(c) Cork the tube securely and centrifuge at 2,250 to 2,750 r.p.m. for 30 minutes. If, at the end of this time, the supernatant is found to be somewhat turbid,

cool the tube for a few minutes under the cold water tap and centrifuge again. Accurate results are obtained only when the supernatant is crystal clear.

(d) Being extremely careful not to disturb the precipitate, gently turn the uncorked tube on its side and permit the supernatant fluid to run off. No attempt should be made to insure complete removal of the fluid at this time.

(e) Centrifuge the uncorked tubes at 2,250 to 2,750 r.p.m. for five minutes. Slowly invert the tubes and let them stand in this position on a layer or two of paper toweling or filter paper for a few minutes.

(f) Add 3.0 ml. of biuret reagent and 3.0 ml. of distilled water to the tubes and shake briskly for 30 seconds. Let stand 15 minutes. Centrifuge to deposit the slight precipitate and decant the supernatant fluid into a cuvet.

(g) Prepare the blank with 3.0 ml. of biuret reagent and 3.0 ml. of water.

(h) After the sample has stood at least 30 minutes, read on the photoelectric colorimeter. Divide the protein value obtained by 3 to obtain the globulin concentration.

9. Beta globulin: Subtract the value for gamma globulin obtained in Step 8h from the value for beta globulin plus gamma globulin obtained in Step 7.

10. Normal values: Obtained from pooled serum samples.

Spectrophotometric Analyses

A Beckman Model B Spectrophotometer[1] was used for all analyses.

Standardization

Standard curves were calculated for human albumin[2] and normal chicken serum. Protein concentrations were determined by the macro-Kjeldahl method.[3]

Using 99.0 per cent pure human albumin (12) approximately 0.8 gram was weighed and diluted to 10 ml. with distilled water. From this solution several dilutions of known protein concentration

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[1] Manufactured by Beckman Instruments, Inc., South Pasadena, California.

[2] Obtained through the courtesy of Dr. Keith McCall, Michigan Department of Health, Lansing, Michigan.

[3] All Kjeldahl analyses were done at the Agricultural Chemistry Laboratory, Michigan State University.

were prepared and analyzed with the spectrophotometer. The results obtained by plotting optical density against protein concentration were used to prepare the standard curve.

A standard curve for pooled normal chicken serum was prepared in a similar manner and used for all readings. The results obtained for the albumin and pooled chicken serum fit the straight-line equation: $Y = a + b(X)$ (Figure 1).

Serum Neutralization Tests

All in vitro serum neutralization tests were performed according to the technic described by Cunningham (23).

Nine-day-old embryonating chicken eggs maintained in an electric forced-draft incubator[1] at 99.5°F. (88°F. wet-bulb thermometer) were used for this test.

The eggs were examined by transillumination in order to determine the site of inoculation. An area devoid of large blood vessels opposite to the embryo and approximately 2 ml. below the base of the air cell was chosen. A hole was drilled through the shell by means of an electric drill without piercing the shell membrane. A second hole was drilled above the air cell.

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[1] Manufactured by James Manufacturing Company, Fort Atkinson, Wisconsin.

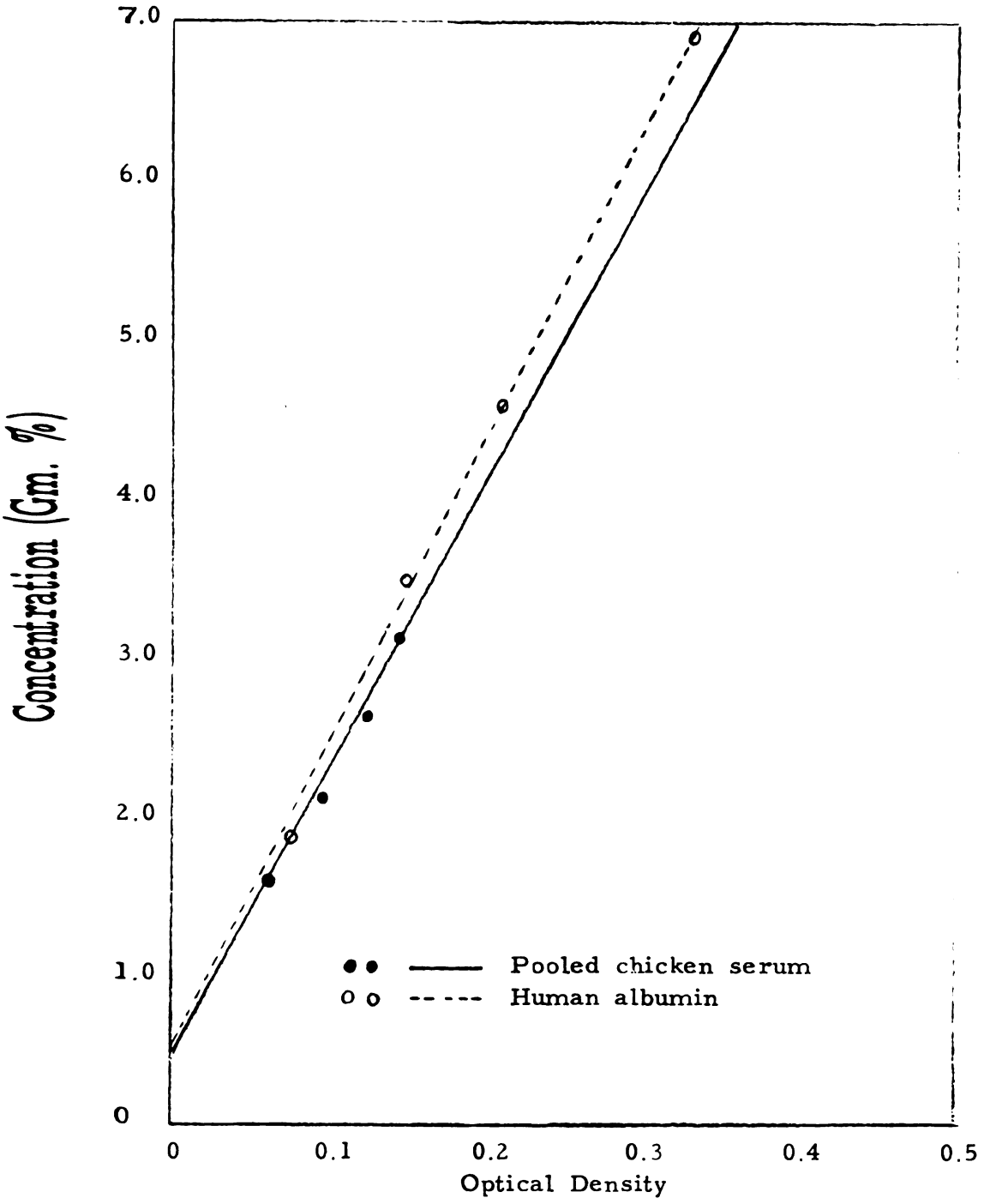

Figure 1. Comparison of total protein of human albumin and pooled chicken serum by the biuret reaction.

Tincture of metaphen was used to paint both holes. The shell membrane under the hole above the air cell was pierced by a sterile teasing needle just before inoculation in order to equalize the pressure produced by the injection of the inoculum into the egg.

The frozen virus-infected allantoic fluid was thawed and centrifuged for five to ten minutes at 2,500 r.p.m. to deposit the amorphous urate material. From the clear supernatant fluid serial tenfold dilutions were prepared in Difco nutrient broth[1] in the proportion of 0.5 ml. of the virus to 4.5 ml. of the diluent.

The serum was passed through a Swinny filter.[2] Serum virus mixtures were prepared separately by mixing equal portions of each virus dilution and undiluted serum.

Quantitative virus titrations were prepared by mixing equal portions of each virus dilution with Difco nutrient broth.

The mixtures were allowed to stand for ten minutes at room temperature and then inoculated into the eggs using 0.1 ml. per egg and five eggs per dilution.

The eggs for the quantitative virus titration were inoculated last to minimize any possible deleterious effect of incubation on the virus.

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[1] Difco Company, Detroit, Michigan.

[2] Manufactured by Becton, Dickinson Company, Rutherford, New Jersey.

After inoculation the holes were sealed with melted paraffin and the eggs reincubated and candled daily for five days. Death occurring during the first eighteen hours was attributed to trauma or other unspecific causes. These eggs were not counted in the final calculations.

The 50 per cent endpoint formula of Reed and Muench (82) expressed as LD_{50} was used to evaluate all titrations. The LD_{50} neutralization index ($LD_{50}NI$) was the difference between the reciprocal of the virus and the serum titers. The antilog was the number of neutralizing doses.

RESULTS

Preliminary Experiments

Preliminary experiments were conducted in order to obtain more detailed information about the technic to be used as well as to adjust the method to the present investigation.

Storage of Serum Samples

To provide for replication and further experiments it was desired to store the serum samples for as long as possible without any deleterious change. Storage at -30°C. was chosen to minimize the effects of any possible bacterial contamination that would occur in processing the samples, thus making unnecessary the use of aseptic technics.

To observe any modification that would occur following freezing and thawing, as stated by Moore (76), one portion of the normal serum was frozen and the other portion stored at 4°C. Both samples were analyzed by the spectrophotometer for total serum protein, and no significant differences were observed.

Wavelength

In order to observe if the wavelength suggested by Wolfson et al. (91) fit the present experiment a pooled sample of serum from normal chickens was examined for total protein at different wavelengths.

The results showed that the greatest absorption of light occurred at 540 millimicrons (Table I).

Sodium Sulfite Concentration

In choosing the technic of Wolfson et al. (91) for fractionation of the serum samples in this investigation, some facts were taken into consideration (20, 21, 64, 74). The method of Howe (55) employed slow filtration which required more than twelve hours. Separation of the fractions in the chosen technic was made by centrifugation as a time-saving procedure. The principle of Kingsley (63), who employed ether to decrease the density of globulin precipitated by sodium sulfate, was used. The application of the same principle in the use of sodium sulfite for determination of albumin was of no value. Addition of a surface active agent such as Span 20 permitted separation of globulin and did not affect the albumin values.

TABLE I

ABSORPTION OF LIGHT AT DIFFERENT WAVELENGTHS

Wavelength (millimicrons)	Optical Density	
	Serum I[1]	Serum II[2]
400	0.14	0.25
450	0.08	0.13
500	0.17	0.32
525	0.22	0.42
540	0.23	0.45
560	0.23	0.43
600	0.17	0.31
625	0.11	0.40
540	0.23	0.45

[1] Pooled chicken serum: Kjeldahl, 4.26 gram per cent; spectrophotometry, 4.40 gram per cent.

[2] Human serum: Kjeldahl, 8.50 gram per cent; spectrophotometry, 8.85 gram per cent.

Wolfson et al. (91) modified this original method for gamma globulin by diluting the serum with ammonium sulfate 1:25. In this manner a higher yield of gamma globulin was obtained as compared to using undiluted serum. No correlation was found between immunologic and electrophoretic estimation of gamma globulin (56). Jager et al. (58) found sodium sulfite the best method for albumin determination after magnesium sulfate.

Leland (67) applied the same technic and found that with rat serum the best concentration of sodium sulfite for albumin determination was 24.0 per cent instead of 28.0 per cent, as suggested by Wolfson et al. (91). This was determined by electrophoretic measurements.

Using a pooled sample of normal chicken serum the percentage of albumin was determined with concentrations of sodium sulfite varying from 20.0 to 30.0 per cent. The same serum was analyzed electrophoretically and it was observed that the solution suggested for human serum was of equal value for chicken serum (Figure 2).

Experimental Results

In order to minimize the variations encountered in the serum components at the several sampling periods when expressed as grams per cent, all results have been analyzed on the basis of per cent total

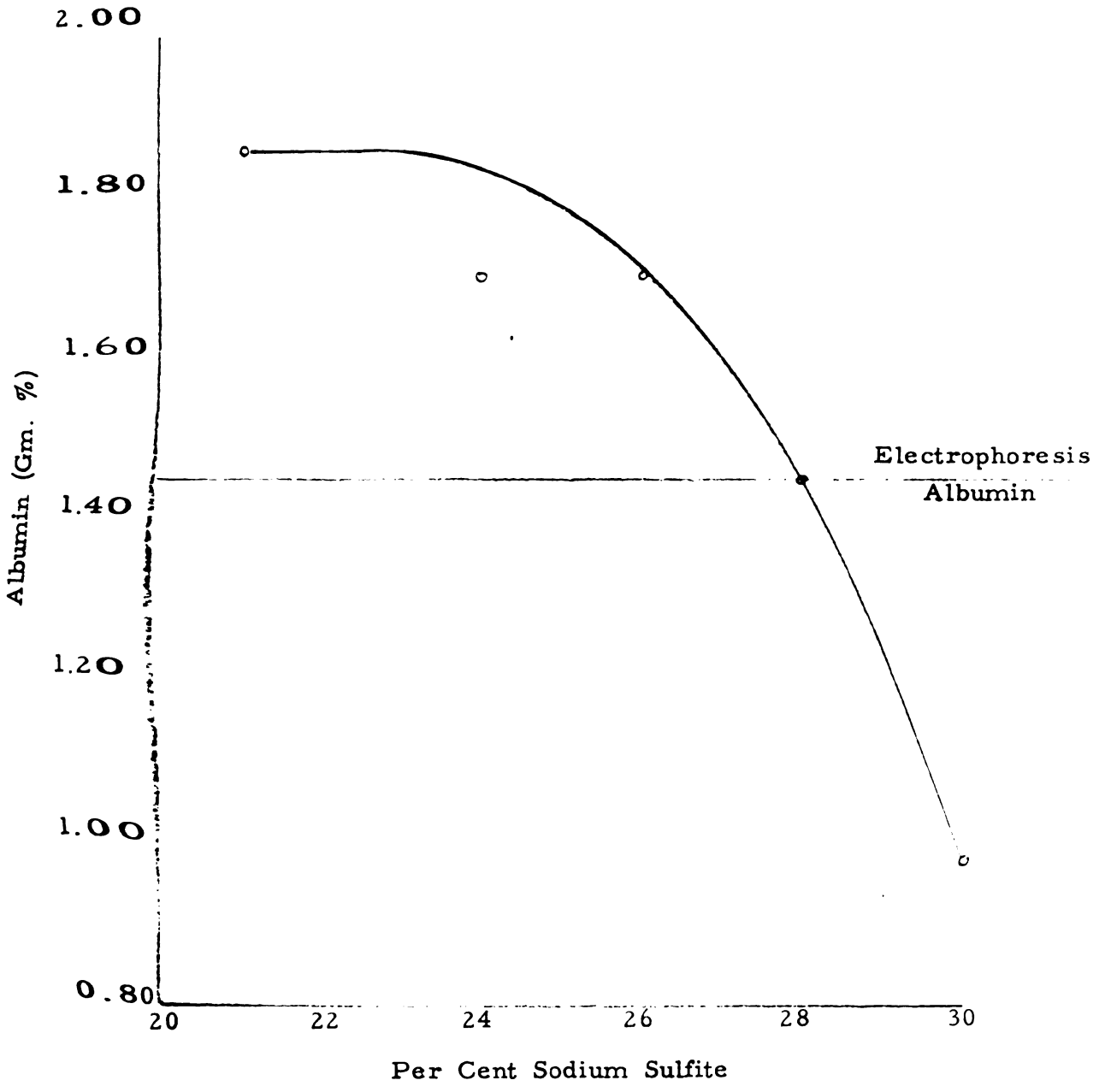

Figure 2. Selection of sodium sulfite concentration for albumin determination.

protein of the sample. This permits a more valid interpretation of the relative per cent serum components.

Individual Sera

Spectrophotometric results

Spectrophotometric results obtained for the individual sera can be seen in Tables II, III, IV, V, and VI, and in Figures 3, 4, 5, 6, and 7.

Total protein. The results obtained with sera from chickens 464 and 469 showed a slight increase in total protein during the period of their participation in the experiment. The others showed a marked variation and with chicken 470 a decrease was observed.

The initial values for all chickens ranged from 3.60 to 3.95 grams per cent protein. At the seventh week the total protein had increased to 3.87 grams per cent with chickens 464 and 466. These values remained constant at the twelfth and twentieth weeks. With chicken 469 the grams per cent total protein was 3.95 at the seventh week and 4.60 at the sixteenth and twentieth weeks. The reverse occurred with chickens 468 and 470, in which the initial protein values were 3.95 and 3.70 grams per cent, respectively. At the seventh week these values had decreased to 3.58 and 3.50 grams per cent protein.

TABLE II

SPECTROPHOTOMETRIC VALUES FOUND FOR CHICKEN 464 IN DIFFERENT PERIODS OF TIME

Item	Time Interval (weeks)					
	0	3	5	7	10	12
Grams Per Cent Protein						
Total protein	3.60	3.60	3.14	3.87	2.90	3.87
Albumin	2.12	1.85	1.30	1.65	1.30	2.03
Total globulin	1.48	1.75	1.84	2.22	1.60	1.84
Alpha globulin	0.38	0.65	0.93	1.10	0.55	0.55
Beta globulin	0.30	0.24	0.11	0.41	0.37	0.55
Gamma globulin	0.80	0.86	0.80	0.71	0.68	0.74
A/G ratio	1.43	1.06	0.71	0.74	0.81	1.10
Relative Per Cent Serum Components Based on Total Protein						
Albumin	58.8	51.4	41.4	42.6	44.8	52.4
Total globulin	41.2	48.6	58.6	57.4	55.2	47.6
Alpha globulin	10.6	18.0	29.6	28.4	18.2	14.2
Beta globulin	8.4	6.6	3.5	10.7	12.7	14.2
Gamma globulin	22.2	24.0	25.5	18.3	24.3	19.2

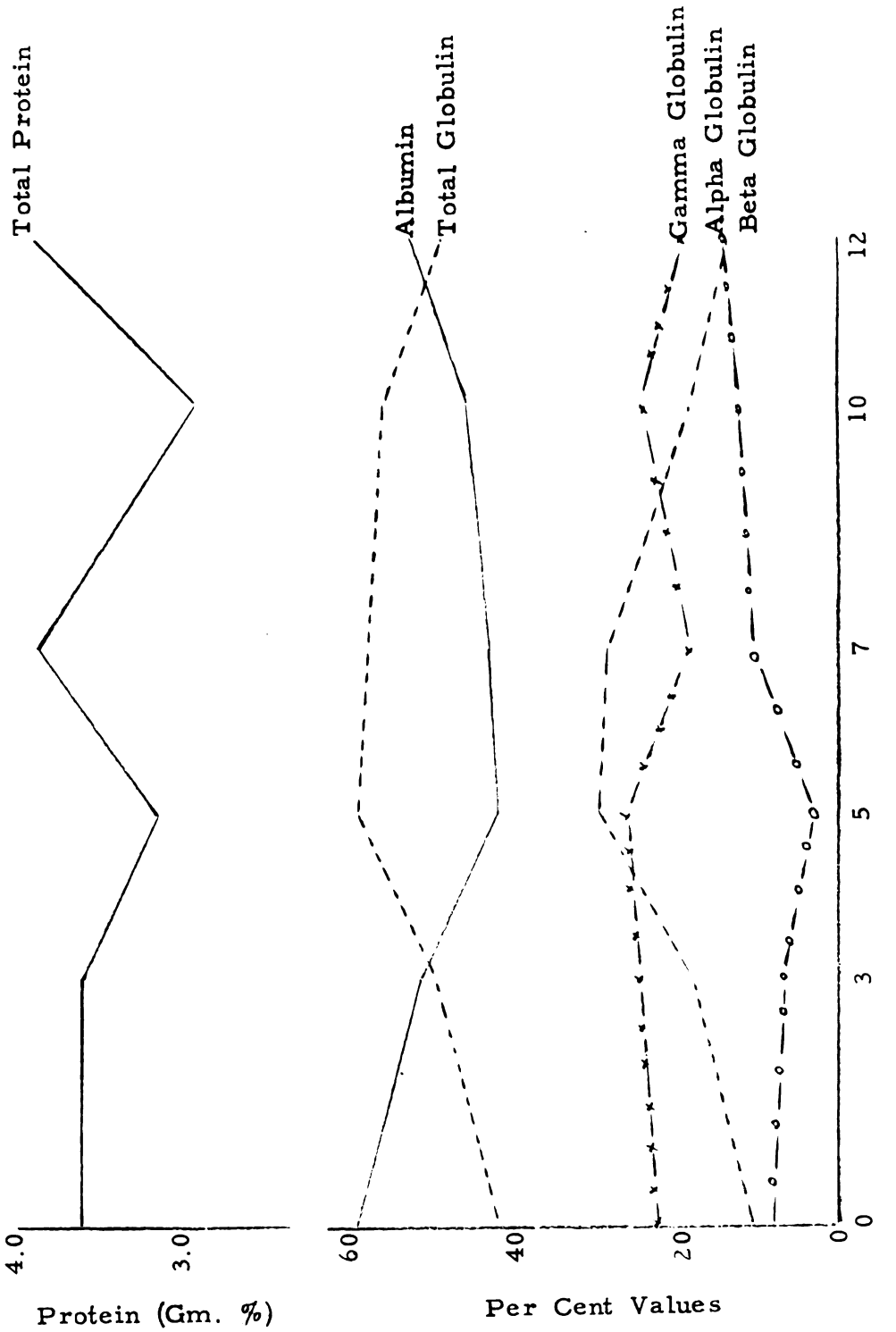

Figure 3. Spectrophotometric values for sera from chicken 464.

TABLE III

SPECTROPHOTOMETRIC VALUES FOUND FOR CHICKEN 466 IN DIFFERENT PERIODS OF TIME

Item	Time Interval (weeks)						
	0	3	5	7	10	12	20
Grams Per Cent Protein							
Total protein . . .	3.80	3.70	3.50	3.87	3.21	3.70	3.87
Albumin	2.58	1.65	1.40	1.42	1.34	1.80	1.00
Total globulin . . .	1.22	2.05	2.10	2.45	1.87	1.90	2.87
Alpha globulin . .	0.27	0.93	1.10	1.33	0.77	0.78	1.80
Beta globulin . . .	0.40	0.27	0.17	0.30	0.35	0.32	0.27
Gamma globulin .	0.55	0.85	0.83	0.82	0.75	0.80	0.80
A/G ratio	2.11	0.80	0.66	0.58	0.71	0.94	0.34
Relative Per Cent Serum Components Based on Total Protein							
Albumin	67.9	44.5	40.0	36.7	41.7	48.6	25.8
Total globulin . . .	32.1	55.5	60.0	63.3	58.3	51.4	74.2
Alpha globulin . .	7.1	25.1	31.4	34.3	24.0	21.1	46.5
Beta globulin . . .	10.5	7.5	4.8	7.7	10.9	8.7	7.0
Gamma globulin .	14.5	22.9	23.8	21.3	23.4	21.6	20.7

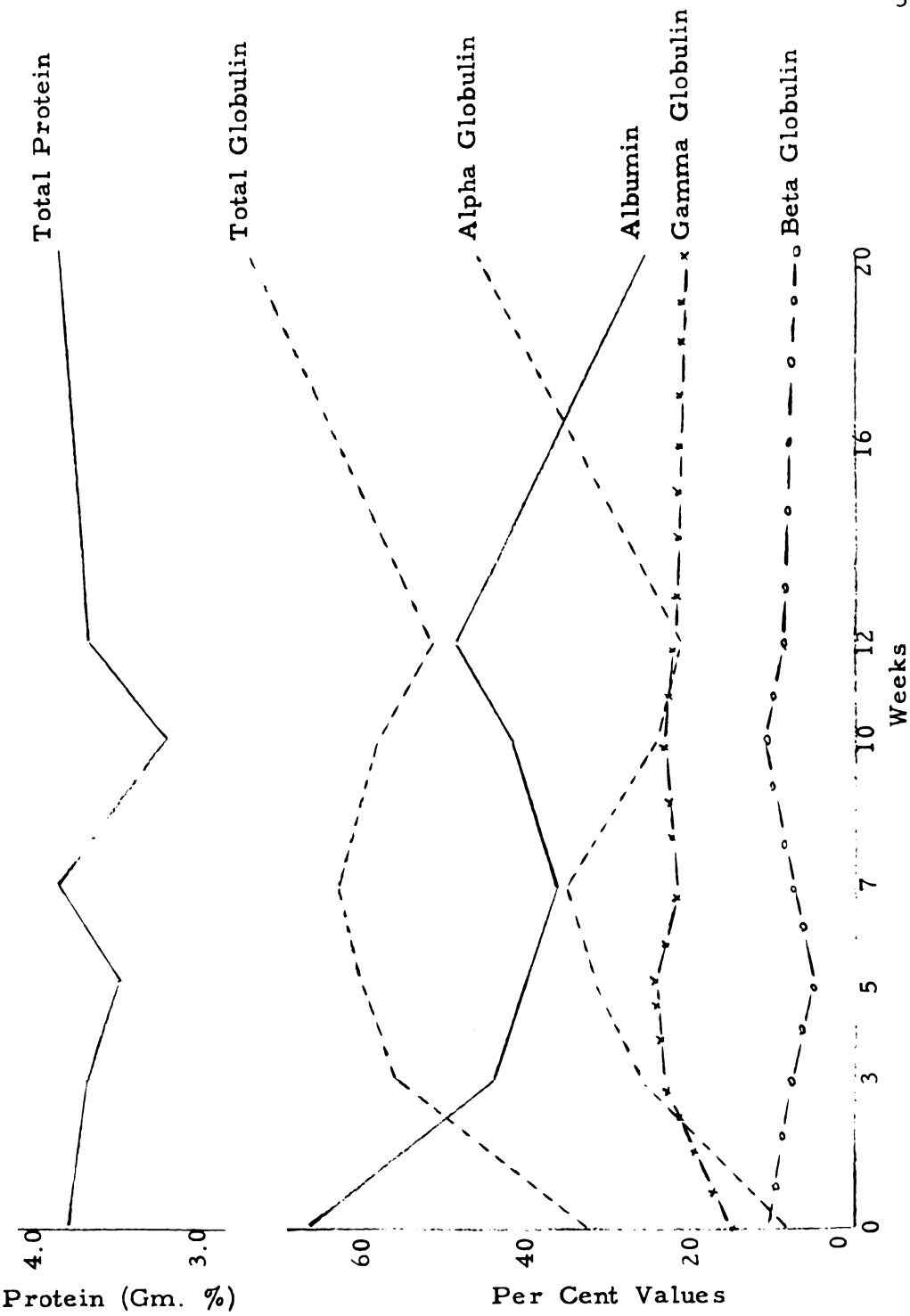

Figure 4. Spectrophotometric values for sera from chicken 466.

TABLE IV

SPECTROPHOTOMETRIC VALUES FOUND FOR CHICKEN 468 IN DIFFERENT PERIODS OF TIME

Item	Time Interval (weeks)					
	0	3	5	7	10	12
Grams Per Cent Protein						
Total protein	3.95	3.70	3.50	3.58	3.70	3.50
Albumin	2.03	1.65	1.65	1.60	1.45	1.85
Total globulin	1.92	2.05	1.85	1.98	2.25	1.65
Alpha globulin	0.47	0.75	0.35	0.63	0.95	0.38
Beta globulin	0.80	0.44	0.60	0.55	0.50	0.53
Gamma globulin	0.65	0.86	0.90	0.80	0.70	0.74
A/G ratio	1.05	0.80	0.89	0.81	0.64	1.12
Relative Per Cent Serum Components Based on Total Protein						
Albumin	51.4	44.5	47.1	44.7	39.1	52.6
Total globulin	48.6	55.5	52.9	55.3	60.9	47.4
Alpha globulin	11.9	20.3	10.1	17.8	25.7	10.8
Beta globulin	20.2	11.8	17.1	15.3	13.5	15.5
Gamma globulin	16.5	23.4	25.7	22.2	21.7	21.1

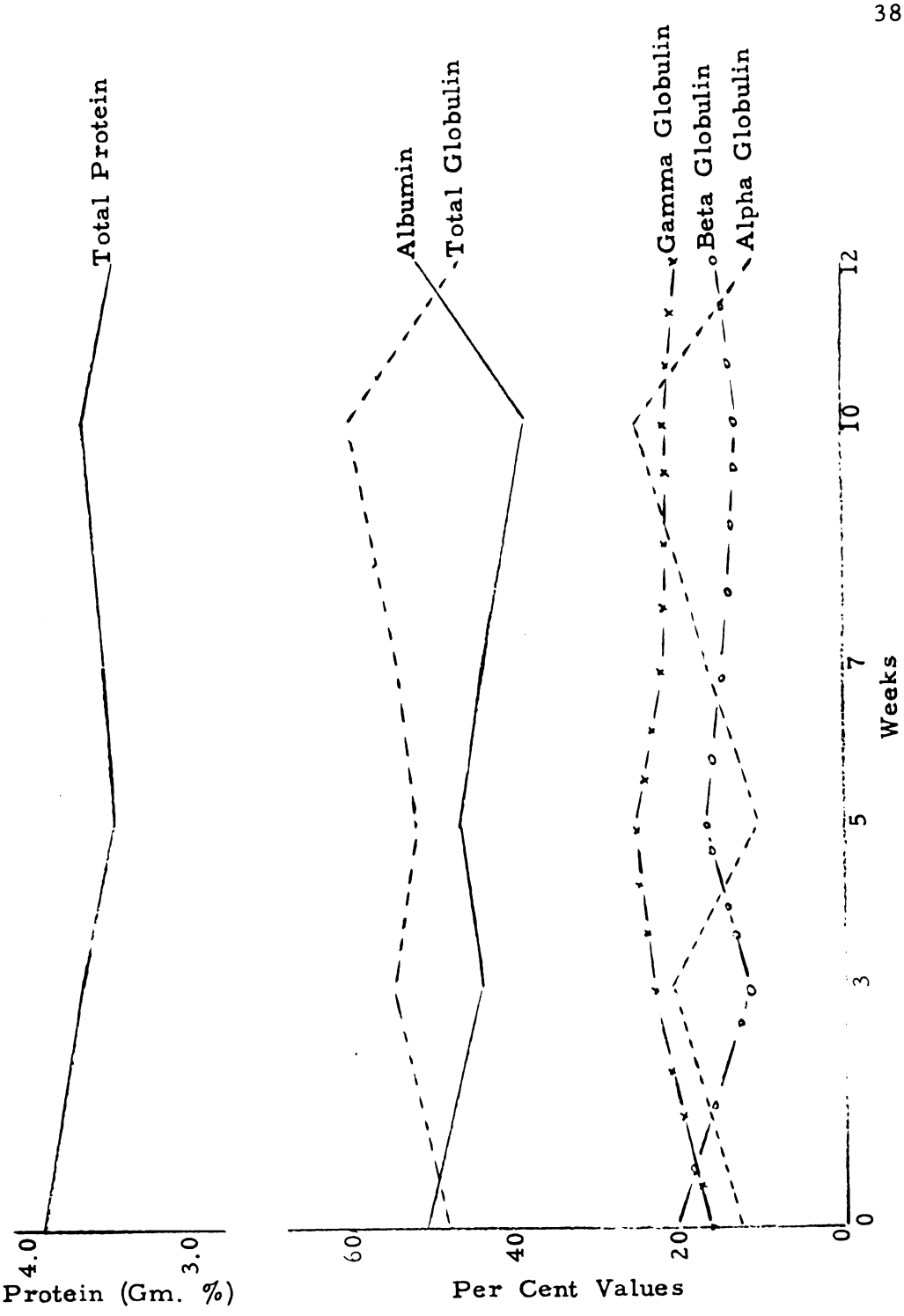

Figure 5. Spectrophotometric values found for sera from chicken 468.

TABLE V

SPECTROPHOTOMETRIC VALUES FOUND FOR CHICKEN 469 IN DIFFERENT PERIODS OF TIME

Item	Time Interval (weeks)							
	0[a]	3	5	7	10	12	16	20
Grams Per Cent Protein								
Total protein . . .	-	3.80	3.40	3.95	3.70	3.87	4.60	4.60
Albumin . .	-	1.85	1.58	1.65	1.44	2.05	2.03	1.47
Total globulin . . .	-	1.95	1.82	2.30	2.26	1.82	2.57	3.13
Alpha globulin . . .	-	0.90	0.65	1.00	1.24	0.90	0.37	1.23
Beta globulin . . .	-	0.25	0.37	0.53	0.26	0.12	1.40	1.07
Gamma globulin . . .	-	0.80	0.80	0.77	0.76	0.80	0.80	0.83
A/G ratio . .	-	0.94	0.86	0.72	0.64	1.12	0.79	0.47
Relative Per Cent Serum Components Based on Total Protein								
Albumin . .	-	48.6	46.4	41.7	38.9	53.0	44.1	32.0
Total globulin . . .	-	51.4	53.6	58.3	61.1	47.0	55.9	68.0
Alpha globulin . . .	-	23.7	19.1	25.3	33.5	23.2	8.1	28.4
Beta globulin . . .	-	6.7	10.9	13.4	7.1	3.2	30.4	21.9
Gamma globulin . . .	-	21.0	23.6	19.6	20.5	20.6	17.4	17.7

[a] Lost.

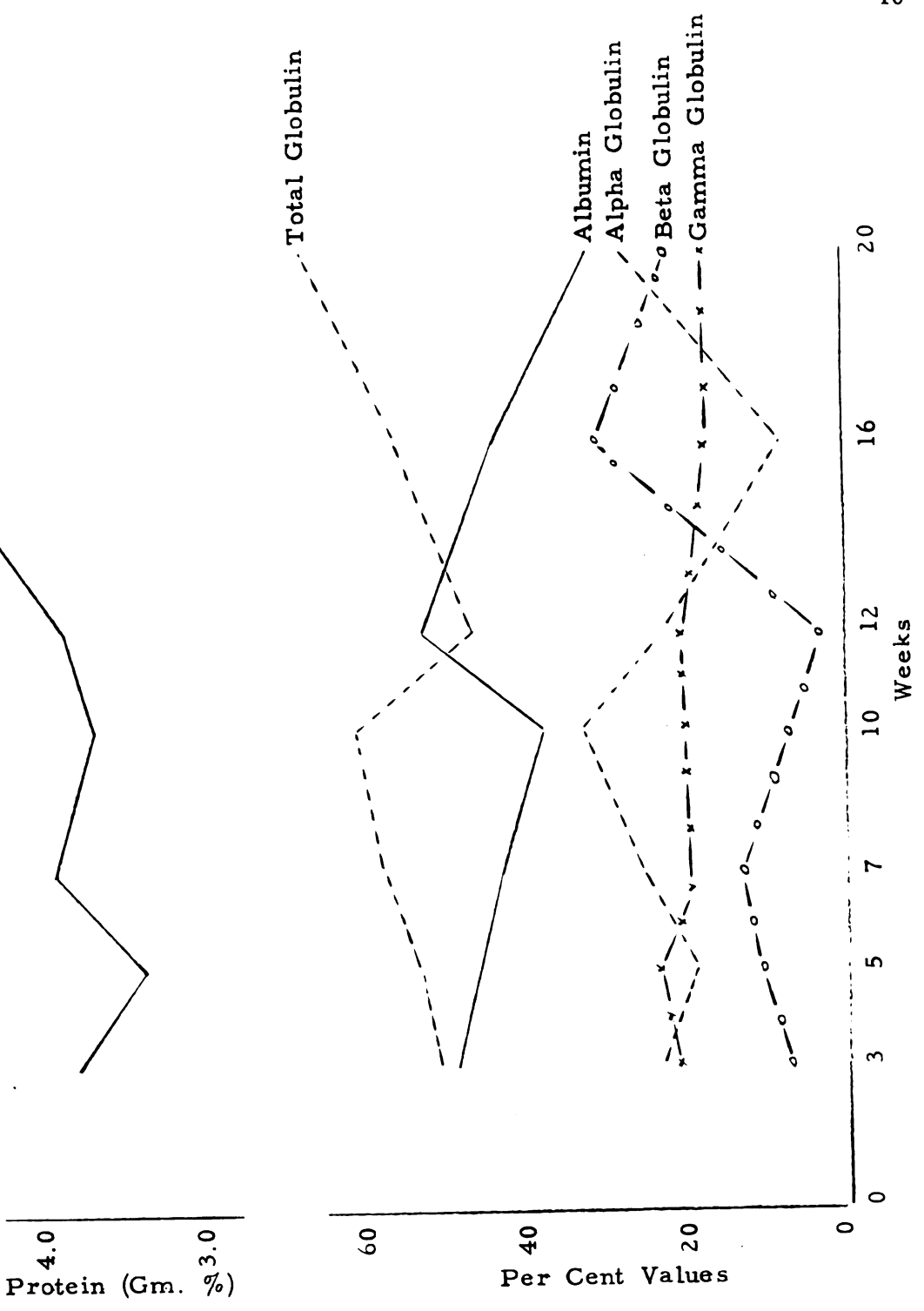

Figure 6. Spectrophotometric values found for sera from chicken 469.

TABLE VI

SPECTROPHOTOMETRIC VALUES FOUND FOR CHICKEN 470 IN DIFFERENT PERIODS OF TIME

Item	Time Interval (weeks)				
	0	3	5	7	10
Grams Per Cent Protein					
Total protein	3.70	3.87	-	3.50	3.55
Albumin	1.85	1.65	-	1.30	1.55
Total globulin	1.85	2.22	-	2.20	2.00
Alpha globulin	0.90	0.85	-	1.15	1.10
Beta globulin	0.15	0.42	-	0.23	0.20
Gamma globulin	0.80	0.95	-	0.82	0.70
A/G ratio	1.00	0.89	-	0.59	0.77
Relative Per Cent Serum Components Based on Total Protein					
Albumin	50.0	42.6	-	37.1	43.4
Total globulin	50.0	57.4	-	62.9	56.6
Alpha globulin	24.3	21.9	-	32.8	31.0
Beta globulin	4.1	11.0	-	6.7	5.9
Gamma globulin	21.6	24.5	-	23.4	19.7

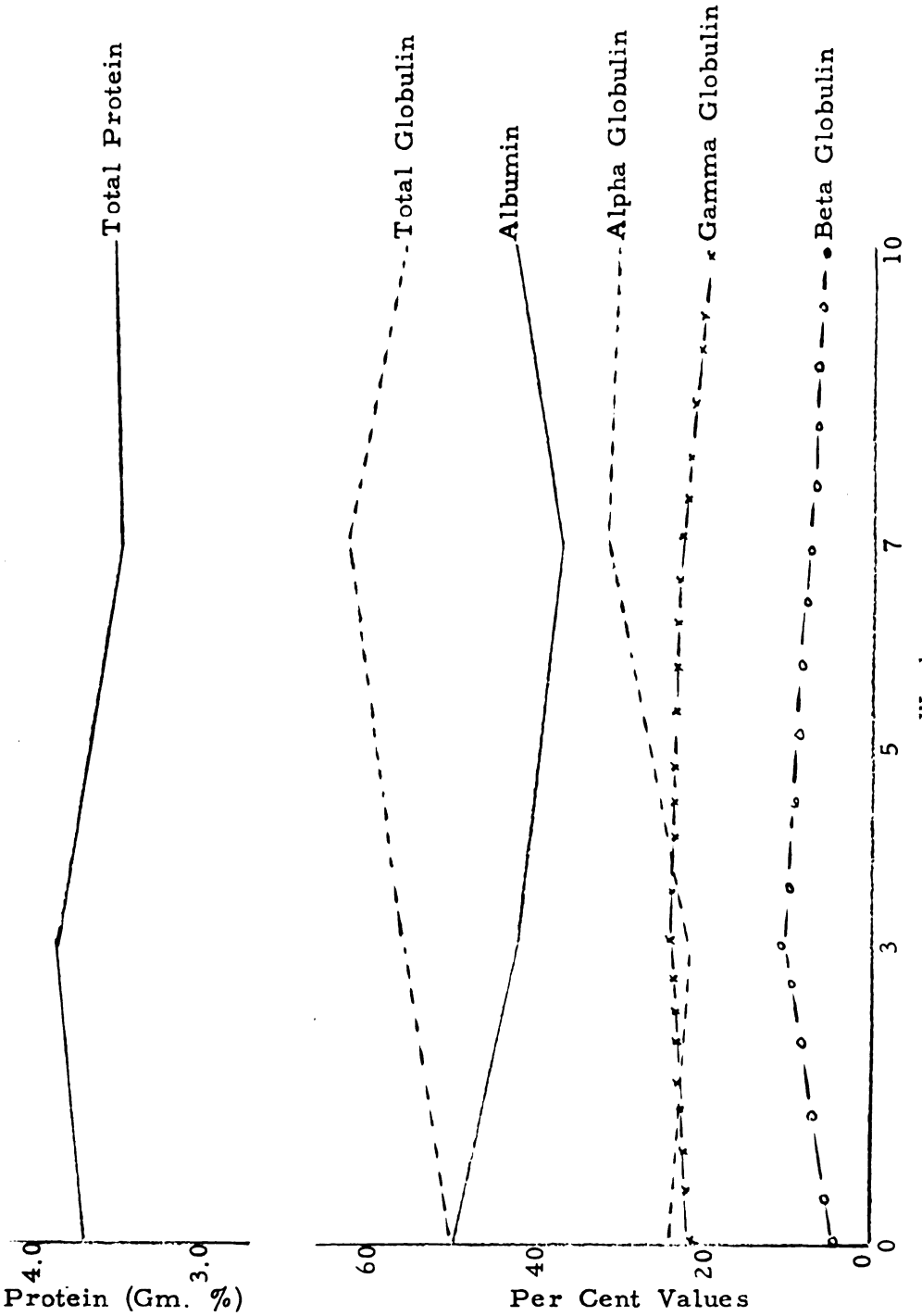

Figure 7. Spectrophotometric values found for sera from chicken 470.

At the twelfth week there was a further decrease to 3.50 grams per cent for chicken 468.

Variations that occurred during the intervening periods did not significantly alter the general trend.

Albumin. Initial values for albumin ranged from 50.0 to 67.9 per cent of the serum total protein. In general there was a decrease in albumin up to the seventh or tenth week followed by an increase at the twelfth week. With bird 464 the lowest value, 41.4 per cent, was obtained at five weeks. Between the tenth and twelfth weeks an appreciable increase to 52.4 per cent was observed.

With chicken 466 a low value of 36.7 per cent for albumin occurred at the seventh week. For chickens 468 and 469 low values of 39.1 and 38.9 per cent, respectively, occurred at the tenth-week period. The initial values for birds 466, 468, and 469 were, respectively, 67.9, 51.4, and 48.6 per cent albumin. The increase observed at the twelfth week was followed by a decrease at the sixteenth week for bird 469. Albumin values at the end of the experiment (20 weeks) were reduced to 25.8 and 32.0 per cent for birds 466 and 469, respectively.

A decrease from 50.0 to 37.1 per cent albumin was observed with chicken 470 between the beginning of the experiment and the

seventh-week period. At the tenth week this value had increased to 43.4 per cent.

Total globulin. With the exception of chicken 469, for which the value for the original sample was not available, all birds showed an increase in total globulin at the third week. This increase was marked with bird 466 for which the total globulin increased from 32.1 to 55.5 per cent. With other birds this increase was not so marked.

After reaching a maximum value of 58.6 per cent at the fifth week, chicken 464 had a decrease in total globulin up to the twelfth week.

With bird 466, a value of 63.3 was obtained at the seventh week, but the maximum of 74.2 per cent occurred at the twentieth week.

Alpha globulin. Examining the initial results, it was found that alpha globulin ranged from 7.1 to 24.3 per cent. Results obtained with individual birds showed some variation. With chickens 464 and 466 a similar pattern was obtained. An increase up to the fifth- or seventh-week period was noted. These values were 29.6 and 34.3 per cent, respectively. From this point through the twelfth week a decrease was observed. At the end of twenty weeks the value for bird 466 was 46.5 per cent. This was more than a sixfold increase from the original value.

The same phenomenon was seen with bird 469. After reaching a 33.5 per cent value at the tenth week, a decrease to 8.1 per cent was observed at the sixteenth week. The final result at the end of twenty weeks was 28.4 per cent. Chickens 468 and 470 showed a large variation. Initial values were 11.9 and 24.3 per cent, respectively. At the tenth week chicken 468 reached 25.7 per cent, whereas the highest value with chicken 470, 32.8 per cent, was obtained at the seventh week. At the tenth week only chicken 470 had a value higher than at the beginning.

Beta globulin. Results found for beta globulin showed a marked variation. Initial values ranged from 4.1 to 20.2 per cent of the total protein.

With bird 464 a decrease was observed at the fifth week when the value was as low as 3.5 per cent. By the twelfth week a substantial increase to 14.2 per cent was recorded.

A similar picture was seen with chicken 466. The only difference was that, after ten weeks, the values were more or less constant at about 8 per cent through the twentieth week.

A decrease from 20.2 to 11.8 per cent beta globulin was observed between zero and three weeks with bird 468. The results at the subsequent periods showed a regular increase and at the end of twelve weeks a value of 15.5 per cent was found.

The highest value for bird 469, 30.4 per cent, was observed at the sixteenth-week period. This was 4.5 times greater than the original value.

With bird 470 a variation occurred. There was an increase from 4.1 to 11.0 per cent between zero and three weeks. After that a slight decrease through the tenth week was observed. Final value was 5.9 per cent, slightly higher than at the beginning.

Gamma globulin. Initial values for gamma globulin ranged from 14.5 per cent for chicken 466 to 22.2 per cent for chicken 464. There was a general increase between the beginning of the experiment through the fifth-week period. The greatest increase occurred with bird 466, from 14.5 to 23.8 per cent.

With birds 464, 466, and 469 a decrease at the seventh week was observed followed by an increase at the tenth week. A subsequent decrease occurred at the twelfth week and extended through the twentieth week with birds 466 and 469. Values obtained at the last bleeding period for these chickens were 19.2, 20.7, and 17.7 per cent gamma globulin, respectively.

Chickens 468 and 470 showed a decrease between three and ten weeks to 21.7 and 19.7 per cent, respectively. Final values for these chickens were 21.1 and 19.7 per cent gamma globulin, respectively.

A/G ratio. The A/G ratio at the beginning of the investigation ranged from 0.94 to 2.11. A general decrease through the fifth and seventh weeks was observed. The most marked decrease was shown with bird 466, in which the ratio changed from 2.11 to 0.58. After this point an increase in A/G ratios was observed through the tenth and twelfth weeks.

With chickens 466 and 469, values as low as 0.34 and 0.47, respectively, were observed at the end of twenty weeks.

Pooled Sera

Spectrophotometric results

The results obtained are shown in Table VII and Figures 8 and 9.

Total protein. At the beginning of the experiment the total protein was 3.73 grams per cent by the macro-Kjeldahl method and 3.60 grams by spectrophotometry. These values remained without marked variation up to the tenth week, when they were 3.6 and 3.4 grams per cent, respectively. While there were slight differences in the values obtained by the two methods at the intermediate periods, there was a definite parallelism with the values obtained by the Kjeldahl method being uniformly higher than those obtained by

TABLE VII

SPECTROPHOTOMETRIC AND MACRO-KJELDAHL DETERMINATIONS ON POOLED SERA

Item	Time Interval (weeks)							
	0	3	5	7	10	12	16	20
	Grams Per Cent Protein							
Total protein (K)[1]	3.73	4.08	3.86	3.99	3.60	3.82	-	4.20
Total protein (S)[2]	3.60	3.87	3.70	3.87	3.40	3.31	4.60	3.87
Albumin	1.84	1.75	1.55	1.65	1.47	1.65	2.03	1.40
Total globulin	1.76	2.12	2.15	2.22	1.93	1.66	2.57	2.47
Alpha globulin	0.65	0.83	1.03	1.05	0.46	0.75	-	1.10
Beta globulin	0.23	0.43	0.37	0.83	0.79	0.21	-	0.63
Gamma globulin	0.88	0.86	0.85	0.84	0.70	0.70	-	0.74
A/G ratio	1.04	0.83	0.72	0.74	0.76	1.01	0.75	0.56
	Relative Per Cent Serum Components Based on Total Protein							
Albumin	51.2	42.7	41.9	42.7	43.4	50.0	44.0	36.2
Total globulin	48.8	57.3	58.1	57.3	56.6	50.0	56.0	63.8
Alpha globulin	18.1	21.4	25.2	24.5	13.5	22.6	-	28.4
Beta globulin	6.3	10.7	10.0	11.1	23.2	6.3	-	16.2
Gamma globulin	24.4	25.2	22.9	21.7	19.9	21.1	-	19.2

[1] Kjeldahl method.

[2] Spectrophotometry.

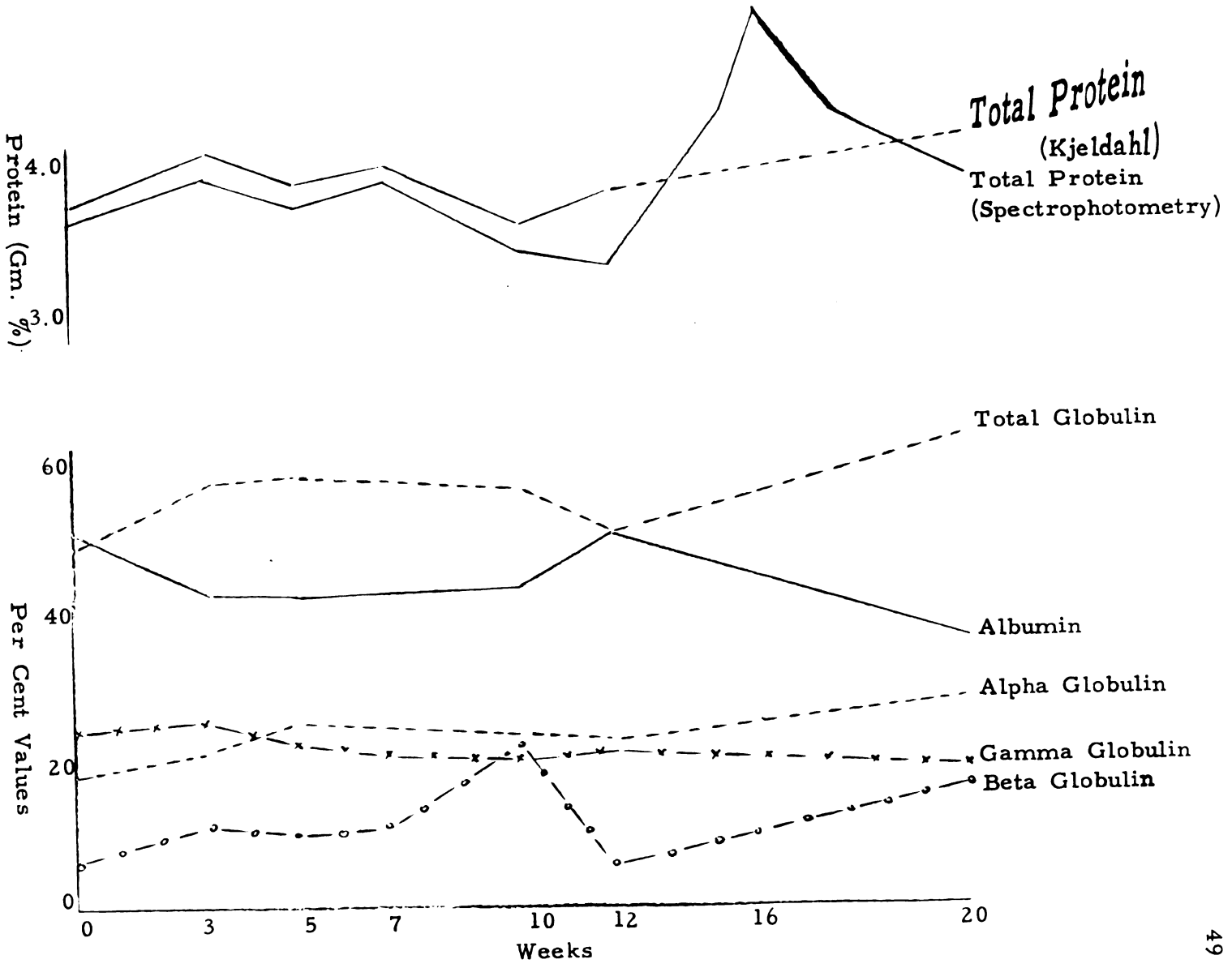

Figure 8. Spectrophotometric values for pooled sera.

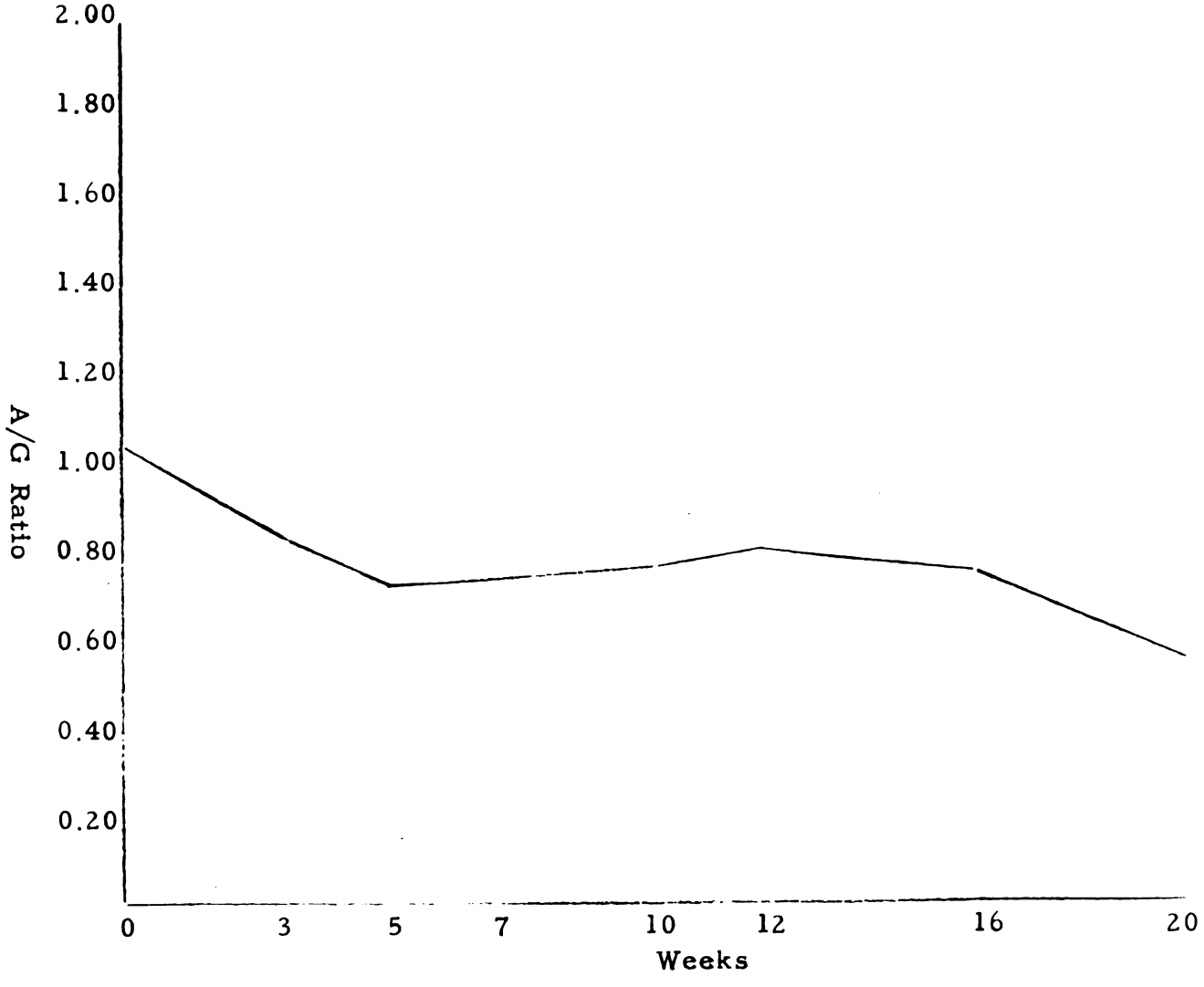

Figure 9. Values found for A/G ratio of pooled sera.

spectrophotometry. At the twelfth week there was a variation in the parallelism in that there was an increase in the value obtained by the Kjeldahl method and a decrease in the value found by spectrophotometry. Unfortunately serum was not available for analysis by the latter method at the sixteenth week and a comparison cannot be made to the spectrophotometric analyses which showed a marked increase in total protein. At the twentieth week the total protein as analyzed by both methods was 4.20 and 3.87 grams per cent, respectively. This was an increase from the beginning of the experiment of 0.47 grams per cent by the Kjeldahl method and 0.27 grams per cent by the spectrophotometric method.

During the course of the investigation there were only two periods at which the total protein was lower than at the beginning. This occurred at the tenth and twelfth weeks as analyzed spectrophotometrically and at the tenth week when analyzed by the Kjeldahl method.

Albumin. The initial value for albumin was 51.2 per cent in relation to the total protein value of the serum. At the third week this value decreased to 42.7 per cent and remained constant until the twelfth week when the value was 50.0 per cent. At the end of the twentieth week the albumin value decreased again to 36.2 per cent which was the lowest value during the course of the investigation.

Total globulin. At the beginning the total globulin was 48.8 per cent. An increase to 57.3 per cent was observed at the end of the third week. This value remained constant until the end of the tenth week when a decrease occurred. There was 50.0 per cent albumin at the twelfth week, but this value increased to a maximum of 63.8 per cent at the twentieth week.

Alpha globulin. Initially the alpha globulin was 18.1 per cent followed by an increase to 21.4 per cent at the third week. This value continued to increase to 25.2 per cent at the fifth week and then a decrease occurred. The lowest value was observed at the tenth week when only 13.5 per cent of the total protein was constituted by albumin. A recovery followed and at the end of the twentieth-week period alpha globulin reached a maximum value of 28.4 per cent.

Beta globulin. From the beginning of the experiment through the tenth week there was an increase of beta globulin from 6.3 to 23.2 per cent.

At the twelfth week the values were 6.3 per cent, followed by an increase to 16.2 per cent at the twentieth week.

Gamma globulin. Gamma globulin values increased from 24.4 per cent at zero weeks to 25.2 per cent at the end of the third week.

Following this period there was a general decrease to 19.2 per cent at the end of the twentieth week.

A/G ratio. A/G ratios showed a direct correlation with the data obtained for albumin and total globulin. The values for these two components showed an indirect relationship at the various sampling periods. The A/G ratio was 1.04 at the beginning of the experiment followed by a decrease to 0.83 at the third week. From this point to the tenth week the ratio was rather constant. At the twelfth week the ratio was 1.01, and at the twentieth week it was 0.56 (Figure 9).

Serum neutralization tests

The results seen in Table VIII and Figure 10 show that the $LD_{50}NI$ for the preinoculation sample was $10^{0.5}$, or three neutralizing doses. This was well within the accepted range of normal chicken serum (22). Following inoculation there was a marked increase in antibodies to the seventh week, when the maximum level of $LD_{50}NI$, $10^{6.5}$, or 3,162,000 neutralizing doses, was obtained. This level was constant through the twelfth week followed by a decline to $LD_{50}NI$ of $10^{5.6}$, or 398,100 neutralizing doses at the sixteenth week, and $LD_{50}NI$ of $10^{5.4}$, or 251,200 neutralizing doses at the twentieth week.

TABLE VIII

SERUM NEUTRALIZATION TESTS ON POOLED SERUM SAMPLES

Time (weeks)	Virus Titer LD_{50}	Serum Titer LD_{50}	$LD_{50}NI$	Neutralizing Doses
0	$10^{-7.0}$	$10^{-6.5}$	$10^{0.5}$	3.1
3	$10^{-6.6}$	$10^{-2.6}$	$10^{4.0}$	10,000
5	$10^{-7.0}$	$10^{-1.0}$	$10^{6.0}$	1,000,000
7	$10^{-6.5}$	$\leq 10^{0}$	$\geq 10^{6.5}$	$\geq$ 3,162,000
10	$10^{-6.5}$	$\leq 10^{0}$	$\geq 10^{6.5}$	$\geq$ 3,162,000
12	$10^{-6.5}$	$\leq 10^{0}$	$\geq 10^{6.5}$	$\geq$ 3,162,000
16	$10^{-6.6}$	$10^{-1.0}$	$10^{5.6}$	398,100
20	$10^{-6.6}$	$10^{-1.2}$	$10^{5.4}$	251,200

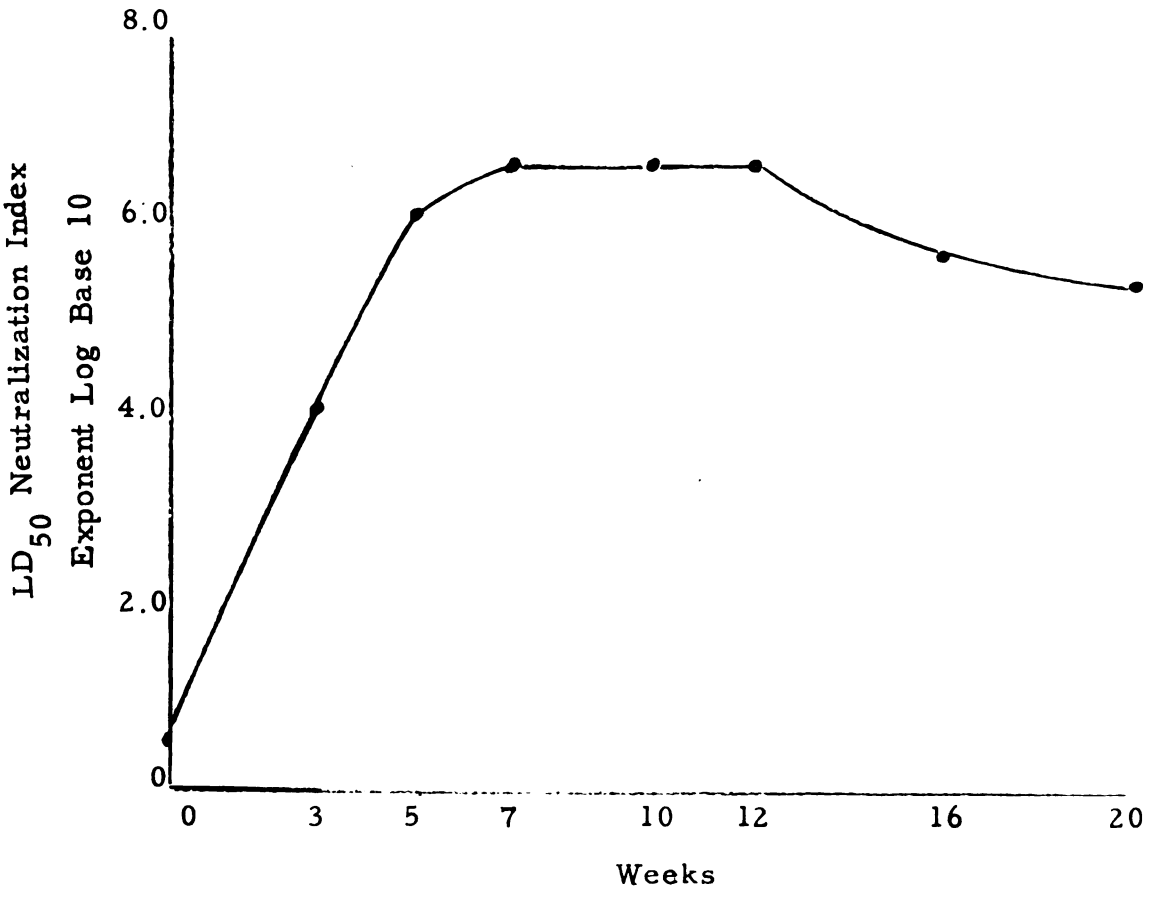

Figure 10. Serum neutralization test of pooled sera.

The antibody response as shown with the serum neutralization test was typical of infection with this virus.

Hemagglutination-inhibition tests of all sera for the presence of Newcastle disease virus antibodies were negative.

DISCUSSION

Chicken serum and plasma have been extensively investigated with the use of the electrophoretic technic (15, 38, 75, 83, 84). The values found for normal chickens showed a wide variation in the relative per cent distribution of the individual serum components.

San Clemente (83), using phosphate buffer at pH 7.7, 0.2 ionic strength, found the following per cent values: albumin, 35.0; alpha globulin, 15.0; beta globulin, 5.0; and gamma globulin, 45.0. A/G ratio found was 0.54.

With veronal buffer at pH 8.6 the electrophoretic analyses of plasma of male chickens were as follows, according to Deutsch and Goodloe (36): albumin, 38.2 ± 1.3 per cent; alpha 1-globulin, 15.8 ± 0.6 per cent; alpha 2-globulin, 7.7 ± 0.5 per cent; beta globulin + fibrinogen + gamma globulin, 37.5 ± 1.3 per cent.

Deutsch et al. (37) used veronal buffer at pH 8.6, 0.1 ionic strength, and found the following average per cent composition: albumin, 46.0; alpha globulin, 22.0; beta globulin, 8.0; and gamma globulin, 24.0.

Sanders et al. (84), using veronal buffer at pH 8.6, 0.1 ionic strength, found for Leghorn chickens the following average per cent

values: albumin, 46.0; alpha globulin, 13.1; beta globulin, 15.7; and gamma globulin, 21.6.

Dimopoullos (38) reported the following values: albumin, 50.5 per cent; alpha, beta, and gamma globulins, 13.5, 11.1, and 24.8 per cent, respectively. A/G ratio found was 1.02.

Results found in the present study using the spectrophotometric technic for pooled sera from adult, single-comb, White Leghorn cockerels prior to infection with IBV were as follows: albumin, 51.1 per cent; alpha globulin, 18.0 per cent; beta globulin, 6.4 per cent; and gamma globulin, 24.5 per cent. A/G ratio was 1.04. These values showed a reasonable agreement with those found by electrophoresis.

Qualitative and quantitative changes in the protein content of serum and its fractions can be induced by physiological as well as pathological conditions such as age, sex, nutritional state, injury, loss of plasma protein, and severity of the disease. Some references will be mentioned to substantiate such assertions. The work of Madden and Whipple (71) is a good review on plasma proteins, their source, production, and utilization.

Modifications through the age have been shown by Brandt et al. (15), who reported a decrease in albumin and an increase in globulin as chickens matured.

Bjørneboe (13) observed that, upon immunization of animals with bacteria, a change in the electrophoretic patterns occurred. There was a direct correlation between globulins and antibody protein.

A direct correlation between increased gamma globulin and antibody titers has been shown by several workers (13, 14, 72, 86, 92). All gamma globulin is not antibodies, nor are all antibodies encountered only in the gamma globulin fraction (40, 61, 62).

It is known that electrophoretic patterns are changed from the normal due to protein depletion (39) or by plasmapheresis (11, 19, 93). The antibody-fabricating mechanism is impaired in hypoproteinemia (11, 18).

In an extensive investigation of sera from normal and IB-infected birds as analyzed electrophoretically it was found that, in chickens bled at weekly intervals, signs of protein depletion were evident by the inversion of the A/G ratios. This was not observed when birds were bled at monthly intervals (38).

Injury is responsible for some changes occurring in the electrophoretic patterns of sera from dogs and goats (47, 48). Leland (67) reviewed this aspect in an investigation of sera from rats immunized against the nematode, Nippostrongylus muris. An increase in beta globulin was found, but this was attributed to trauma in the

lungs due to formation of cysts by the parasite. The lungs were considered to be the probable site of antibody formation and not responsible for the increase in beta globulin.

No specific changes in the electrophoretic patterns of sera were found when birds were subjected to tracheal injury similar to that used for inoculation of IBV into susceptible chickens (38).

From the results obtained during the course of the present investigation the first point to be considered is the slight increase in the total protein as shown by macro-Kjeldahl and spectrophotometric methods. A reasonable agreement was obtained although values by the Kjeldahl method were consistently higher than those obtained with spectrophotometry. Differences between the two methods ranged from 0.17 to 0.51 grams per cent protein, corresponding to 4.5 to 13.3 per cent deviation.

Albumin values decreased, and as a result the A/G ratios were lowered considerably at the bleeding periods subsequent to the exposure of birds to IBV. An increase of globulins with decrease of albumin appears to be the characteristic serum alteration in many diseases (50). A rise in globulins can be produced by: (1) formation of antibodies, (2) alteration of the relative production and utilization of albumin and globulin, and (3) compensatory rise in globulins as an attempt to maintain the osmotic pressure.

The first two conditions have already been discussed. Consideration must be given to the third condition for increase in globulins.

Increase in globulins and a decrease in albumin is somewhat a compensatory mechanism. Albumin is responsible in great part for the osmotic pressure. Osmotic pressure depends upon the number of particles and not the size, and it has been proposed that the globulin concentration compensates for any decrease in albumin.

According to Bjørneboe (13), a definite relationship between albumin and globulin concentration maintains the optimal osmotic pressure. This hypothesis has not been accepted generally (46).

In an endeavor to explain the results obtained it must be emphasized that a large volume of blood (20 ml., or approximately one-fourth of the total blood volume) was removed from each bird at every bleeding period. Although the intervals between bleedings were never less than two weeks, it is possible that this interval might not have been sufficient for the complete restoration of the original balance of the serum components.

Results found with pooled sera when compared with those of individual chickens showed a fair agreement for total protein, albumin, total globulin, gamma globulin, and A/G ratios.

Alpha globulin values for individual birds were quite variable. Results with pooled sera showed a tendency to increase, except between the fifth and twelfth weeks, when a small decrease was observed. Final values were higher than those observed at the beginning of the experiment.

With beta globulin a marked variation was also found. The tendency shown by the pooled sera was an increase up to the tenth week, followed by a sharp decrease at the twelfth week. Values at the end of twenty weeks were higher than the initial figures.

Increase in globulins was due especially to increase in alpha and beta globulins, since with gamma globulin a very small variation was observed.

Results obtained by the serum neutralization test of pooled sera showed a close agreement with those found by other investigators (38, 78, 79). The decrease in antibody titer observed by Page (78) in the eighth week after inoculation was not confirmed.

The highest antibody titer ($10^{6.5}$) occurred at the seventh, tenth, and twelfth weeks after inoculation.

Prior to infection with IBV the $LD_{50}NI$ was $10^{0.5}$, or three neutralizing doses, confirming results found by other investigators (22, 38, 78, 79).

There was no correlation between the changes in serum components and antibody content. When the serum components had returned to preinfection levels the antibody content was at the maximum. This was also observed previously by Dimopoullos (38) with the electrophoresis apparatus.

No definite changes in serum electrophoretic patterns were found in animals immunized with West and Venezuelan equine encephalomyelitis (77) and Japanese B encephalitis (66). Viruses probably behave differently from bacteria in the formation of antibodies.

Determination of antibody content in the fractions of anti-IB serum seems to be highly desirable. Short bleeding intervals and withdrawal of large amounts of blood should be avoided.

SUMMARY

1. Spectrophotometric and serum neutralization studies were performed with sera of normal and IBV-infected adult, single-comb, White Leghorn cockerels.

2. Experimental birds were bled by intracardiac puncture immediately before inoculation with IBV and at three-, five-, seven-, ten-, twelve-, sixteen-, and twenty-week postinoculation intervals.

3. Sera obtained from these chickens were clarified by centrifugation and kept at -30°C. until used.

4. Spectrophotometric analyses were performed with Beckman Model B Spectrophotometer on individual sera before and after chemical fractionation by the method of Wolfson et al., and the following values were determined: total protein, albumin, and alpha, beta, and gamma globulins.

5. Pooled sera from birds bled at the same time intervals were analyzed spectrophotometrically and serum neutralization tests were performed. Macro-Kjeldahl analyses were also made of these pools.

6. A close agreement was observed in the total protein determination by the macro-Kjeldahl and the spectrophotometric

methods. Values obtained with the former method were slightly higher.

7. Results obtained by the use of the spectrophotometer after chemical fractionation showed a fair agreement with results found by electrophoresis.

8. A decrease in albumin and a consequent inversion of the A/G ratio was observed after IBV inoculation. Alpha and beta globulins were specifically responsible for the increase in globulins. Gamma globulin had no appreciable influence, as it was of a relatively constant concentration throughout the experiment.

9. Serum neutralization tests conducted on pooled sera showed typical results following IBV infection. A maximum titer was obtained between seven and twelve weeks after inoculation.

10. There was no correlation between the changes in serum components and antibody content. When the serum components had returned to preinfection levels the antibody content was at the maximum.

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