SEROLOGICAL STUDIES ON AVIAN VISCERAL LYMPHOMATOSIS USING THE COMPLEMENT FIXATION REACTION

By

Robert F. Gentry

A THESIS

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

DOCTOR OF PHILOSOPHY

Department of Bacteriology

Year 1953

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Approved W. L. Stafseth

ABSTRACT

Mortality in chickens, resulting from avian visceral lymphomatosis, is of major economic importance to the poultry industry. Isolation and genetic resistance influence this mortality rate, but are not practical for the widespread control of the disease. The development of a diagnostic test would be a major contribution in this field, and this work was done in an attempt to develop such a technique by use of the indirect complement fixation reaction.

Attempts were made to utilize the technique of Rice (1946) and Mayer (1947), both of whom employed the slope of the hemolytic curve in the calculation of the amount of fixation. The slope value was found to vary greatly, depending on the reagent present, and a technique was developed which eliminated the use of such a value. The amount of fixation was determined in terms of milliliters of undiluted complement necessary for 50 percent hemolysis by plotting the electrophotometer readings against the volume of undiluted complement present. From this the units of complement fixed could be calculated.

Influenza virus was employed for the standardization of the test technique. Rabbits were first used as a source of complement fixing antibodies, but were found unsuitable due to the formation of heterophile antibodies which reacted with heterophile antigen of the chicken serum. The production of these antibodies had been stimulated by heterophile antigen in the influenza virus suspension and had originated from

the embryos used for its propagation. Guinea pigs were found not to produce this antibody and were used for subsequent inoculations.

Test antigens were prepared by extracting normal chicken tissue and tumors of RPL strain 12 visceral lymphomatosis (Burmester et al., 1946). They were used for the production of antiserums in guinea pigs, chickens, and ducks. Infected and normal chicken serums were also used as antigens, both in tests and for inoculations.

The first tests were designed to determine if the agent of the strain 12 tumor could be detected in the chicken serum. Infected chicken serum was used as an antigen and allowed to react with guinea pig antiserums that had been produced by the inoculation of normal and infected chicken serum and normal tissue and tumor extracts. Some reaction took place, but was due to the presence of normal tissue materials and not the result of tumor agent activity.

A direct test, employing tumor antigen and guinea pig antitumor serum was standardized for use in testing chicken antitumor serums for their tumor agent antibody content. A reaction, in excess of that due to the normal tissue materials was noted, apparently due to either tumor cell components or the agent.

Chicken antitumor serums were tested by the indirect technique, but no agent stimulated reaction could be detected. The reaction taking place was found to be due to normal tissue materials. Normal tissue antibodies were adsorbed from the guinea pig antitumor serum with normal chicken red blood cells, but when the adsorbed antiserum was used in direct reactions for the indirect titration of chicken antitumor serums,

no difference was noted from the titers obtained with the untreated antiserum.

The detection of the agent of visceral lymphomatosis or specific antibodies was not possible with the techniques used, and will depend upon the development of more highly purified agent suspensions and the elimination of the normal tissue materials.

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I. INTRODUCTION

Lymphomatosis, a naturally occurring malignant disease of chickens, is characterized by the formation of lymphoid tumors in almost any of the tissues of the bird's body. The lymphoid cells infiltrate the tissues, producing a generalized enlargement of the area and giving it a characteristic grayish color. In some cases nodules are formed which are made up exclusively of the malignant lymphoid cells.

The occurrence of lymphomatosis is widespread throughout the world. The economic loss in this country alone has been estimated to be over $60,000,000 annually (Tenth Annual Report of the Regional Poultry Research Laboratory, 1949). This amount represents the monetary value of the birds that die from the disease. It is only a portion of the total loss involved, since the cost of feed and maintenance up to the time of death must also be taken into consideration. The neural form attacks primarily the younger birds, while the visceral form appears somewhat later, especially during the laying period of pullets, and deaths are continuous thereafter as long as the birds are maintained (Biester and Devries, 1944) (Waters and Prickett, 1946). The losses from this disease are felt in all branches of the poultry industry.

From what has been said, it is evident that the development of a method for the control of lymphomatosis would be of great economic importance. There are at present, two methods for limited control of the disease: breeding for genetic resistance, and isolation. In breeding for genetic resistance, chickens are selected on the basis of the performance of their ancestors (Johnson and Wilson, 1937) (Waters, 1945) (Hutt and Cole, 1947).

The second method of control is isolation. By segregating a group of birds so that they have no contact with other chickens at any time during their life, a marked reduction, and in some instances, the complete elimination of disease manifestations, can be brought about. This procedure consists of selecting eggs from certain hens, and incubating, hatching, and rearing the resultant chickens in complete confinement and under rigid quarantine (Waters and Prickett, 1944). This procedure, as well as the method of selective breeding, would not be practical for widespread control of the disease.

Transmission of the agent causing lymphomatosis in its various forms appears to occur by the following methods: from parent to offspring through the egg (Cottral, 1949), by direct bird to bird contact (Waters, 1947) (Waters and Bywaters, 1949), and possibly by indirect contact (Waters, 1947) (Hutt and Cole, 1947).

Indirect contact by means of feces and other body excretions may play an important role (Biester and Devries, 1944). The only excretions, however, which have been definitely shown to transmit the infection, are nasal and tracheal washings (Cottral, 1952).

The methods of transmission mentioned previously all indicate the presence of an infective agent. The genetic resistance exhibited by certain inbred lines is apparently due to a factor which prevents the manifestation of infection in the form of tumors, but not one that prevents the entrance of the agent into the chicken's body.

The first isolation of an agent capable of producing lymphomatosis was achieved by Furth and Breedis (1933). More recently Burmester (1947a), using high speed centrifugation, concentrated the agent of a

lymphoid tumor now designated as RPL* Strain 12 (Burmester et al., 1946). He also demonstrated filtrable agents of other lymphoid tumor strains designated as RPL 16, 18, 19, 20, and 21 (Burmester, 1947b). The tumor material, usually liver, was minced, suspended in saline and centrifuged to sediment the cellular components. If the supernatant fluid was inoculated into young chicks, usually at one day of age, no immediate response was noted. However, after a period of approximately 75 to 160 days, tumors, indistinguishable from those occurring naturally, were produced (Burmester et al., 1946). Similar results were obtained following inoculation of tumor filtrates which had been prepared by passage through a bacteria retaining filter (Burmester et al., 1946). It was thus demonstrated that an agent, capable of passing a bacteria retaining filter, was present, and that this agent would produce tumors indistinguishable, both grossly or microscopically, from the naturally occurring tumor of visceral lymphomatosis.

With the establishment of the presence of a causative agent which could be concentrated by centrifugation, its use in the development of a test was considered. It had been shown by Burmester (1947c) that the lymphoid tumor cells stimulated the production of a factor considered to be an antibody, which had a strong cytotoxic action toward subsequently inoculated tumor cells. This factor was found in the serums of chickens which had survived a tumor cell inoculation or had been inoculated repeatedly with a suspension of non-viable tumor cells. Serum containing this factor showed a toxic effect upon tumor cells both in vivo and

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* Regional Poultry Research Laboratory

in vitro. Even though there was apparently an antibody produced which was active against the cells, it could not be used to identify the agent. A practical test would necessitate the production of an antibody capable of reacting with the agent and the resulting reaction made detectable by some type of indicator system. The complement fixation test was selected to be used in an attempt to identify the occurrence of such a reaction, and a report of the findings when such a test was used constitutes the basis of this thesis.

II. REVIEW OF LITERATURE

The phenomenon of bacteriolysis, due to the action of complement upon a bacterial antigen-antibody complex, may be observable due to the breakdown of the bacteria. Many antigens, however, which are capable of inducing the production of specific antibodies and forming an antigen-antibody complex which will react with complement, are not of sufficient size to allow the easy detection of changes which occur and indicate a reaction with complement. A reaction of this type, therefore, must be made evident by the use of an indicator system. This system must consist of an antigen and specific antibody which will, when allowed to combine, react readily with complement. Also, the resulting reaction must be easily detected. Red blood cells and an antibody specific for them are used for this purpose. The antibody, specific for the red cells, is called hemolysin.

The red blood cells, hemolysin, and complement constitute the indicator system of the complement fixation test. When placed together, the red cells and hemolysin combine and with the addition of complement, hemolysis will occur, as is illustrated in Figure 1a. The solid line enclosing the names of the reagents is meant to indicate that they react together. When a dotted line is used, the absence of a reaction is indicated.

When a specific antigen-antiserum mixture is prepared, the complex formed will, with few exceptions, unite with complement. Such a reaction inactivates the portion of the complement taking part in this union and it is said to be fixed. If excess complement is present, the uncombined

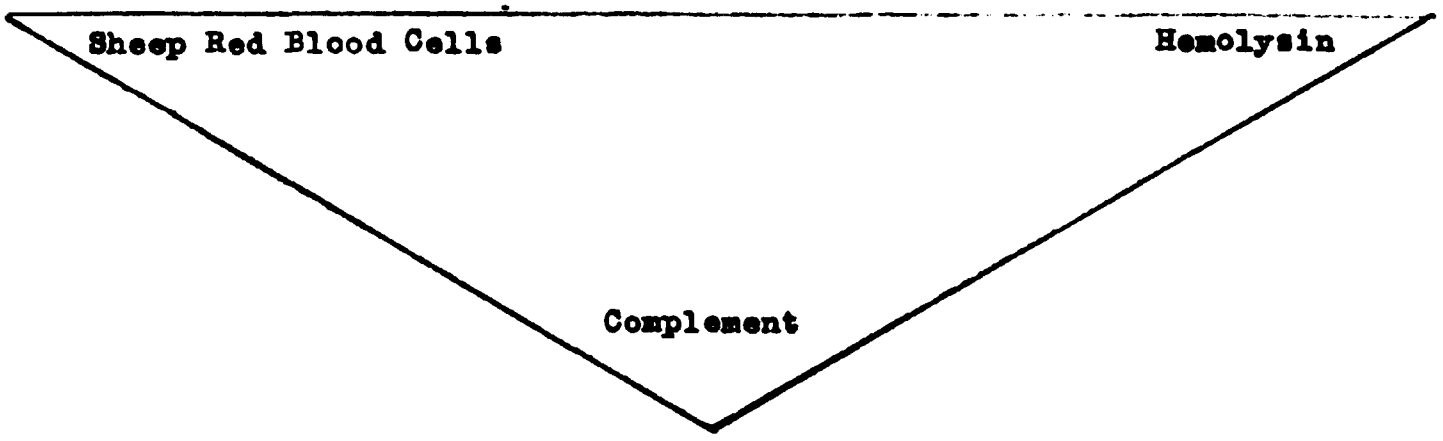


Figure 1a. Diagram of the Indicator System of the Complement Fixation Reaction

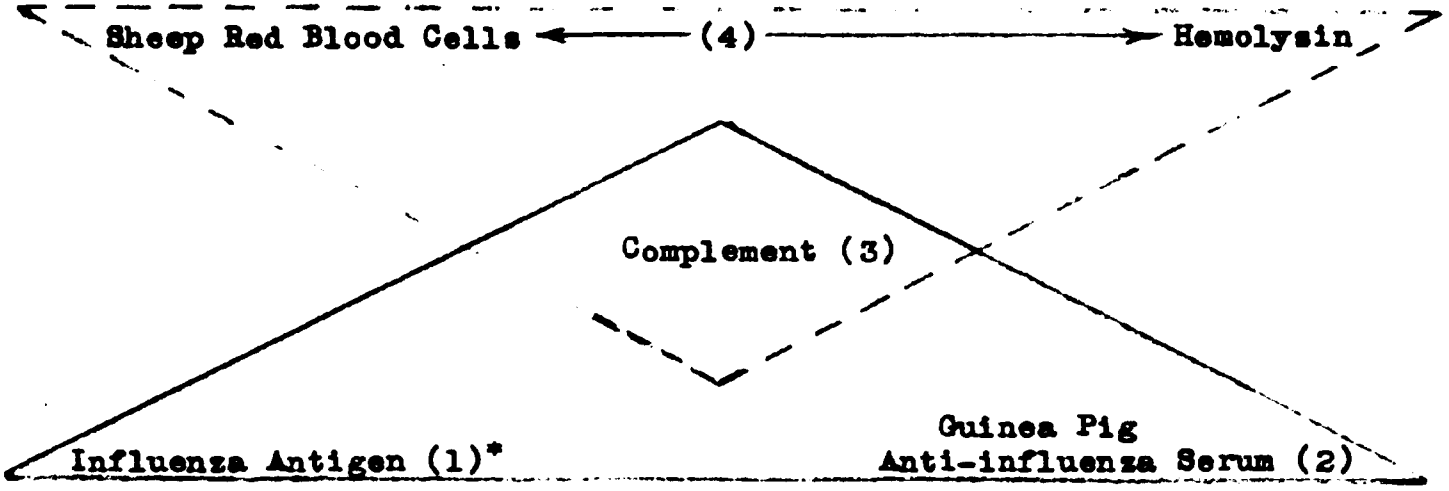


Figure 1b. Diagram of a Positive Complement Fixation Reaction

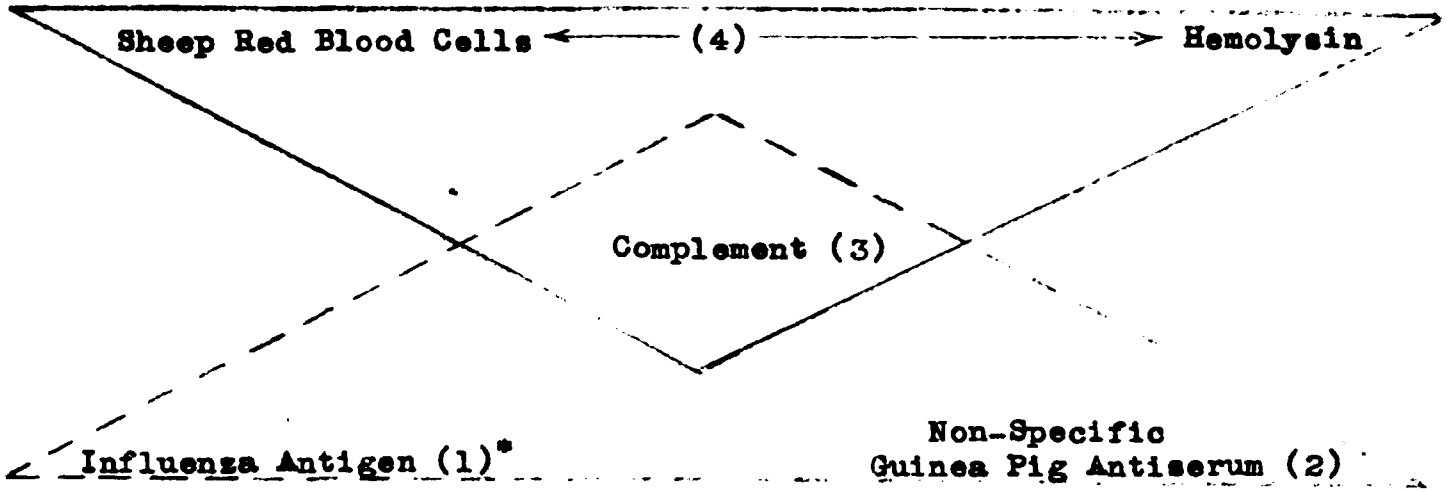


Figure 1c. Diagram of a Negative Complement Fixation Reaction

* ( ) is the order of addition of reagents

portion remains active and is capable of reacting with additional amounts of the same or a different antigen-antibody complex. In order to determine if any complement remains free after the antigen-antibody complex being tested has had time to react, the red blood cell-hemolysin mixture is added. If the test system has fixed all of the complement, no action on the red cells occurs and the test is called positive (Figure 1b). It must be assumed that the amount of complement present in a test, such as the one shown in Figure 1b, had been adjusted so that all would be fixed by the antigen-antibody complex of the test system. If this had not been done, the excess complement would react with the indicator system, and the test system reaction could not be detected. If, on the other hand, the antigen and antibody of the test system are not specific or there is insufficient antibody present, no detectable effect on the complement will take place. This will leave complement free to react with the red cell-hemolysin complex (indicator system) and hemolysis will be noted (Figure 1c). In this case the test is called negative. Influenza virus and guinea pig anti-influenza serum are used in the illustrations.

This basic procedure for the detection of the fixation of complement was developed by Bordet and Gengou (1901), (Gengou, 1902) and is often referred to as the Bordet-Gengou phenomenon. Soon after Bordet and Gengou's experiments, Citron (1906) showed that bacterial extracts could be successfully substituted for bacterial suspensions in these reactions. Wasserman, while working with Neisser (Wasserman et al., 1906a) and Schucht (Wasserman et al., 1906b), developed a method for the diagnosis of syphilis in man by complement fixation, which is known as the

Wasserman reaction. This was the first widely used diagnostic test employing the phenomenon of complement fixation.

In complement fixation reactions which use 100 percent hemolysis as the end point, as is the case with the Wasserman reaction, the degree of hemolysis is determined by holding the tube containing the material in front of a bright light and observing the turbidity. The variations in turbidity are graded as 4+, 3+, 2+, 1+, ±, and -, which correspond to approximately 0, 40, 80, 90, 97, and 100 percent hemolysis.

When amounts of complement are plotted against the percentages of hemolysis produced by them, a sigmoid curve is formed. Larger amounts of complement are required per unit change of hemolysis as the hemolysis approached either 0 or 100 percent. It has been shown (Brooks, 1918) that the differences in the susceptibilities of the red cells to lysis by complement are responsible for the deviation from a straight line relationship and are due to various kinetic factors involving the concentrations of erythrocytes, antibody, and complement, and their respective speeds of interaction (Mayer et al., 1948a), and the susceptibilities in the red blood cell population to lysis by complement (Waksman, 1949).

In the range between 20 and 80 percent hemolysis, there is practically a straight line relationship between the amount of complement and the hemolysis produced, and within this range, where a small amount of complement produces a large change in the degree of hemolysis, more accurate measurement of the amount of complement present can be made.

A highly quantitative test was developed using 50 percent hemolysis as the end point (Wadsworth, 1939, Maltaner and Maltaner, 1935, Thompson

and Maltaner, 1940, Wadsworth et al., 1931, 1938a, 1938b, and 1938c), and utilization made of an instrument such as an electrophotometer or a spectrophotometer (Friedewald, 1943, Mayer et al., 1946b). This type of technique was used in the studies reported in this thesis.

The complement fixation test, as it has been outlined, could not be used for the detection of antibodies in chicken serum because the reaction of these antibodies with specific antigen does not form a complex capable of fixing complement (Rice, 1948b). However, chicken antiserum will combine with specific antigen and the antigen involved in such a reaction is no longer free to react with other antiserum. For example, if chicken anti-influenza serum is mixed with influenza antigen, they will combine but will have no effect on the complement. If guinea pig anti-influenza serum is added, it can not combine with the antigen and no complex capable of fixing complement will be formed. If there were no specific antibodies in the chicken serum, the guinea pig anti-influenza serum would react with the antigen and the complement would be fixed as is shown in Figure 1b. The extent to which the antigen-guinea pig antiserum complex is inhibited measures the antibody titer of the chicken antiserum, and since this measurement is made indirectly, the technique has been called the "Indirect Complement Fixation Test."

The actual mechanics of the reaction may be more clearly understood with the aid of diagrams such as those shown in Figures 2a and 2b. Figure 2a represents a positive indirect reaction. The chicken anti-influenza serum contains antibodies which will combine with the influenza antigen; therefore, their symbols are enclosed in a solid line to indicate this reaction. The guinea pig anti-influenza serum, when added, can

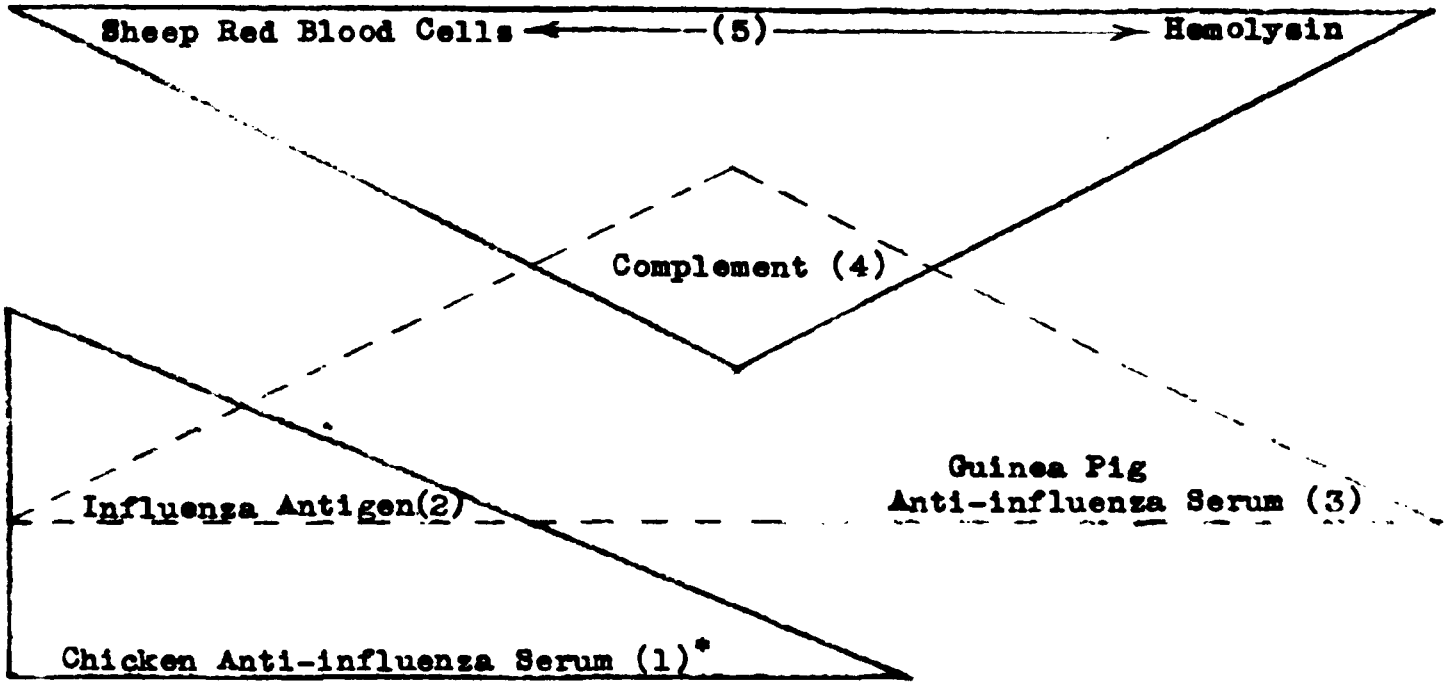


Figure 2a. Diagram of a Positive Indirect Complement Fixation Reaction

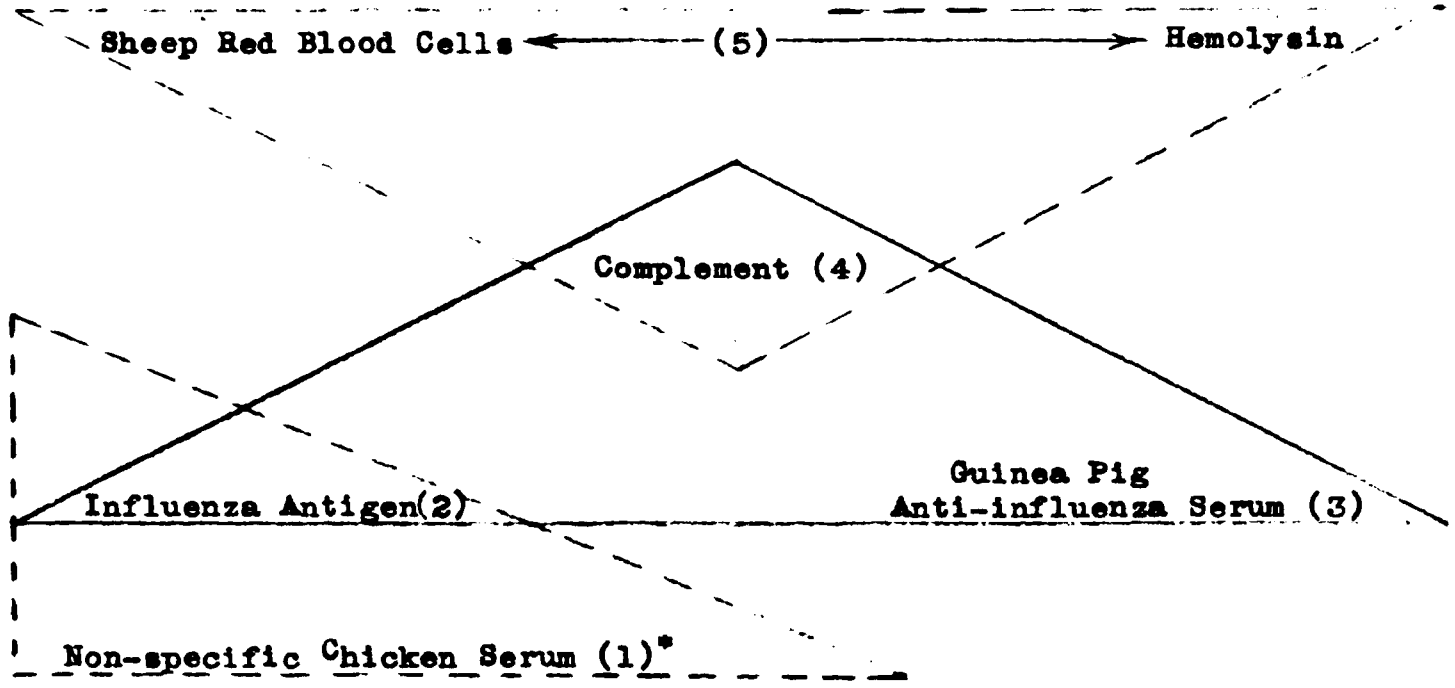


Figure 2b. Diagram of a Negative Indirect Complement Fixation Reaction

* ( ) is the order of addition of reagents

not react, since all the antigen has been bound by the chicken antibodies. The complement is thus not affected, and when the indicator system is added, the complement is free to act upon it and hemolysis occurs. In Figure 2b, a negative indirect reaction is shown. The chicken serum does not contain antibodies specific for the influenza antigen and no reaction occurs. When the guinea pig anti-influenza serum is added, the antigen reacts with it and the complex formed fixes the complement. With the addition of the indicator system, no complement is available and no hemolysis is seen. These illustrations assume that all or none of the various reagents are involved in the respective reactions. In actual practice, the proportionate part of each reagent which enters into the reaction is quantitatively measured.

The use of a serodiagnostic test for visceral lymphomatosis must be of a nature which will allow the detection of the agent causing the disease, and not merely a demonstration of the presence of tumor tissue. In human cancer, the detection of the presence of tumor tissue would be of value. As has been pointed out by Maver (1944), such a test could be used for the differentiation of cancer and tuberculosis or to indicate the absence of metastases or residual growths following surgery. Also, it would provide a method for detecting growths while they were still in the operable stage. Many procedures have been used in attempts to develop such a test. Reviews by Davidsohn (1936) and Maver (1944) are available which show that the tests for the various types of tumors in animals and humans may be divided into three groups: (1) those which measure the comparative concentrations of the constituents of the blood, or the chemical or physical characteristics dependent upon these

constituents, (2) those which depend upon a specific antigen-antibody reaction, and (3) those which compare the proteolytic or lipolytic activities of the serums. Only tests of the type mentioned in group 2 are of interest here. These tests, however, are designed to allow the identification of a specific material which is a component of the tumorous tissue, such as purified protein fractions (Kidd, 1942) and not an agent.

Many of the tumors of animals have been shown to be caused by an agent, presumably a virus. The first demonstration of an agent of this type was made by Rous (1911), while working with sarcoma in fowls. Other animal tumors, such as the adenocarcinoma of frogs (Lucke', 1938), benign papillomas of the skin of western cottontail rabbits (Shope, 1933), the mouse mammary carcinoma (Bittner, 1936), and Leukosis of fowls (Ellerman and Bang, 1908) are known to be produced by virus-like agents. Serodiagnostic methods have been attempted for all these various types of tumors and are discussed in a review by Maver (1944). Only the tests concerned with the various forms of the avian leukosis complex (Biester and Devries, 1944) will be mentioned here.

The classification "leukosis" includes all the various forms of the disease listed under the avian leukosis complex. The early studies were made on the erythroblastic form. In this disease, the immature forms of erythrocytes become the dominant blood cell. Thomsen et al. (1953) used a ground suspension of these cells as an antigen for complement fixation studies. They reported fixation in about 20 percent of the serums under study. These serums were from chickens which had been inoculated with a suspension of the neoplastic cells. The fixation observed was not constant on repetition and may have been due to factors other than a specific

antigen-antibody reaction, since antibodies formed in chickens are not of a type capable of reacting with complement (Rice, 1948b).

Kabat and Furth (1940) attempted precipitin and complement fixation studies on the sedimented agents of fowl erythroleukosis and sarcoma, but were unable to disclose any specific antibody in immune serums prepared from tumor extract and leukotic serum. They attributed their failure to demonstrate difference by immunological methods between the materials sedimented from tumorous and normal spleen extracts by high speed centrifugation, to the presence of large amounts of "normal heavy material" in the virus preparations. They concluded that if agents for these conditions were present, they were there either in small amounts or they were less effective antigens than the normal heavy substance. Kabat and Furth (1941) did demonstrate, however, that the agent of the Rous sarcoma produced neutralizing antibodies in the rabbit. Similar sediments from normal chicken spleen produced no neutralizing antibodies, and the complement fixing antibodies produced by both materials were unrelated to the neutralizing antibodies. Similar results were obtained by Foulds (1937) and Lee (1942), using the serums of ducks, turkeys and chickens which had been inoculated with lymphoid and myeloid tumor extracts.

Pollard et al. (1943) obtained complement fixation reactions with one form of the avian leukosis complex, namely, hemocytoblastosis. The malignant blood cells were ground in a Ten Broeck grinder, centrifuged at low speed, and the supernatant fluid was used as the antigen. Serums from adult turkeys and guinea fowls which had been inoculated with whole blood from normal chickens and from chickens showing lesions of various

forms of lymphomatosis and leukemic conditions were tested against the antigen. No reaction was observed when the serums of the animals receiving normal chicken blood were used, but all the serums of those receiving blood from infected chickens gave positive reactions. Since the antigen was an extract of malignant white blood cells or their precursors, and the positive antiserums were produced by the inoculation of whole blood containing the same type of cells, the positive reactions were apparently not due to an agent responsible for the condition, but rather to components of the malignant cells and a specific antibody produced by the inoculation of an extract of the same type of cells. It indicated, however, that the tumor cells formed in these related conditions were similar and contained common components distinguishable from normal blood cells.

Kissling (1947) described a rapid slide test using stained, washed, formalinized lymphocytes which agglutinated in the presence of serum from a lymphomatosis positive chicken. Investigations of this test by Darcel (1950) showed that whereas a high correlation was obtained with birds showing advanced cases, healthy birds in a flock also showed a high number of positive reactions. The lymphocytes were not found to be necessary in the reaction because the dye and serum would react similar to the reaction between horse serum and isamine blue, the phenomenon described by Dean (1937).

The use of a test, particularly complement fixation, in efforts to detect the agent of visceral lymphomatosis, has not been reported. The segregation of the agent in sufficiently pure form to permit its use as an antigen in the production of antiserum, and the development of a test

which would allow the identification of either the agent of visceral lymphomatosis, or specific antibodies in an infected chicken, were the aims of the work reported in this thesis.

III. MATERIALS AND METHODS

a. Complement:--It has been shown by Wadsworth (1947) that complement can be preserved for an indefinite period of time by freezing. Pooling of the serums of a large number of guinea pigs would afford a constant supply of complement and eliminate the necessity of collecting and processing small batches at frequent intervals.

Approximately 300 young guinea pigs, weighing 800 to 1000 grams each, were secured and bled by cardiac puncture. Eight to 10 milliliters of whole blood was taken from each animal, placed in a small vial, and allowed to clot in an ice water bath. The vials were then placed in a refrigerated centrifuge and spun for five minutes at 1500 g to constrict the clot. The samples showing hemolysis, due to the mechanical breakdown of the red blood cells, were discarded. The serums of the remaining samples were then decanted into a large flask which was immersed in an ice water bath and rotated continually to insure complete cooling and mixing. The resultant pool was transferred to small vials in 1, 3, and 5 milliliter amounts and sealed with rubber stoppers. Each vial, immediately after sealing, was placed in a wire basket in a mechanical freezer where it was frozen and stored at -35° C.

The guinea pigs surviving the first bleeding (approximately 200) were held for seven days and bled a second time. The serums obtained from this second bleeding were handled in the same manner as outlined for the first group. Approximately 2000 milliliters of guinea pig serum was obtained from the two collections, and served as a common source of complement for all the tests.

b. Hemolysin:--The detection of a specific reaction between an antigen and an antiserum, by the complement fixation test, is dependent upon the demonstration of the fixing or inactivation of the complement. As was mentioned previously, the hemolysis of the sheep red blood cells is used as an indicator of this reaction; however, the presence of the red blood cells alone is not enough. The complement must have an antigen-antibody complex to act upon, and the sheep red blood cells act as the antigen for such a complex. When inoculated into rabbits, the sheep red blood cells stimulate the production of a specific antiserum which is called hemolysin (Kabat and Mayer, 1948).

Commercial antisheep hemolysin was used for the tests reported here. It had been preserved by the addition of an equal volume of glycerol, and when kept in a refrigerator at 2-4° C, the titer remained constant for an indefinite period of time.

c. Sheep red blood cells:--A special flask was prepared for the collection of the sheep red blood cells. It consisted of a 500 milliliter Erlenmeyer flask fitted with a number 6, two-hole, rubber stopper. Through one hole was placed a three-inch piece of 6 millimeter glass tubing which was plugged loosely with cotton and acted as an air vent. The other hole was fitted with a six-inch piece of the glass tubing to which was attached a heavy walled rubber tube approximately 18 inches in length. The blunt end of a 16-gauge California type bleeding needle was inserted into the free end of the rubber tube. The beveled end of the needle, which protruded from the tube, was pushed through the center of a square piece of gauze and inserted into a test tube which was of sufficient size to fit snugly over the end of the rubber tube. The gauze

prevented the rubber tubing from adhering to the glass wall of the test tube during sterilization. A screw clamp on the rubber tube completed the collection flask.

The preservative for the sheep red blood cells was a modified Alsever's solution (Bukantz *et al.*, 1946) consisting of:

Dextrose	2.05 grams
Sodium citrate	0.80 grams
Sodium chloride	0.42 grams
Citric acid	0.055 grams
Distilled water	100 milliliters

This amount was sufficient to preserve 100 milliliters of blood; it was placed in a collection flask and steam sterilized. The sterilization had to be limited to 15 minutes at 121° C (steam at 15 pounds pressure) to prevent caramelization of the solution.

Sheep were made available at the Michigan Department of Health laboratories, Lansing, Michigan for the collection of blood. An area approximately three inches square over the jugular vein was clipped, lathered, shaved, washed with water, rubbed dry, and painted with iodine. After careful removal of the test tube and gauze, the needle was inserted into the jugular vein and 100 milliliters of blood allowed to flow into the flask. The flask was agitated continually to insure complete mixing of the blood and preservative solution. When 100 milliliters of blood had been obtained (determined by a mark previously placed on the side of the flask) the screw clamp was tightened and the needle removed. Before storage, the preserved cells were transferred to sterile glass bottles and sealed with rubber stoppers. Sheep red blood cells, when collected

and stored in this manner, remained in good condition for six to eight weeks, after which time a portion of them started to break down and a new batch had to be secured.

d. Diluents:--The components of the complement fixation test vary in their activity and must be diluted so they will be present in optimal concentrations. Also, after the antigen, antiserum, and complement are present, the volume must be made constant so that when the sensitized red blood cells are added, a uniform volume and red cell concentration are obtained. The diluent selected for use in these tests was described by Mayer *et al.* (1946a). They had shown that the addition of cations, especially those of magnesium and calcium, and to a lesser extent, those of cobalt and nickel, had a marked enhancing action on the hemolytic activity of complement. Veronal buffer, containing magnesium and calcium cations, was prepared as follows:

Sodium chloride	85.0 grams
5,5-diethyl-barbituric acid	5.75 grams
Na 5,5-diethyl-barbiturate	3.75 grams
$MgCl_2 \cdot 5H_2O$	1.02 grams
$CaCl_2 \cdot 2H_2O$	0.224 grams
Distilled water	2000 milliliters

The acid was first dissolved in 500 milliliters of hot distilled water and the other components then added. The resulting solution was sterilized by steam at 121° C for 15 minutes. This constituted a stock solution which was diluted with four parts of distilled water before use. Due to the presence of the cations, the solution has been called "Ionic buffer" and will be referred to as such.

The ionic buffer was used as the vehicle for all of the components of the tests. This included the diluting of the antigens, antiserums and complement to the proper concentrations, addition to the test system for standardization of the volume, dilution of the hemolysin, and the final suspension of the red blood cells. The only place where this diluent could not be used was in the washing of the red blood cells. The red cell preservative employed as an anticoagulant, sodium citrate, which prevented clotting by its action on the calcium of the blood plasma (Dukes, 1942). If the ionic buffer was used for washing the cells, an excess of calcium cations was available which overcame the action of the sodium citrate and clotting occurred. Therefore, a solution which did not contain an excess of these cations had to be used for the washing process. A phosphate buffer (Kent, 1946) was selected for this purpose and prepared as follows:

Sodium chloride	170.0 grams
$KH_2 PO_4$	2.4 grams
$Na_2H PO_4$	11.3 grams
Distilled water	1000 milliliters

The components were mixed and heated slightly to facilitate dissolving and the mixture sterilized by steam at 121° C for 15 minutes. This served as a stock solution and was diluted with 19 parts of distilled water before use. After several washings with this solution, the plasma components responsible for the clotting were sufficiently removed so that ionic buffer could be placed with the red cells for the preparation of the standard suspension.

Titrations of complement were conducted to determine the extent of the enhancing action of the calcium and magnesium ions. This was done by titrating complement with ionic buffer as the diluent and comparing the titer to titrations in which phosphate was employed. As will be seen in Table I, less than half as much complement was needed to produce 50 percent hemolysis when the ionic buffer was used as when phosphate buffer was present, which agreed with the findings reported by Mayer et al. (1946a).

e. Electrophotometer:--It has been shown by Kent et al. (1946) and Mayer et al. (1946b) that the measurement of the amount of hemolysis by the spectrophotometric or electrophotometric technique is very accurate when 50 percent hemolysis is used as the end point. A Fisher model AC electrophotometer was used for these determinations.

A straight line should be obtained by plotting the log scale readings from the drum dial of the electrophotometer for varying concentrations of a given solution. Since the material measured is a solution of hemolized sheep red blood cells, a similar solution was prepared by adding 2.5 milliliters of washed sheep red blood cells to 122.5 milliliters of distilled water. The resulting solution was then used for the preparation of a series of hemoglobin concentrations, considering the original solution as representative of 100 percent hemolysis.

Three filters were available for use in the electrophotometer. They had their peak transmittance at different wave lengths, namely 425, 525, and 590 millimicrons. To determine which of these was most applicable, the various solutions, ranging in hemoglobin concentration from 10 to 100 percent, were tested against each of the three filters. The values

TABLE I

THE EFFECT OF CALCIUM AND MAGNESIUM CATIONS ON THE TITER OF COMPLEMENT

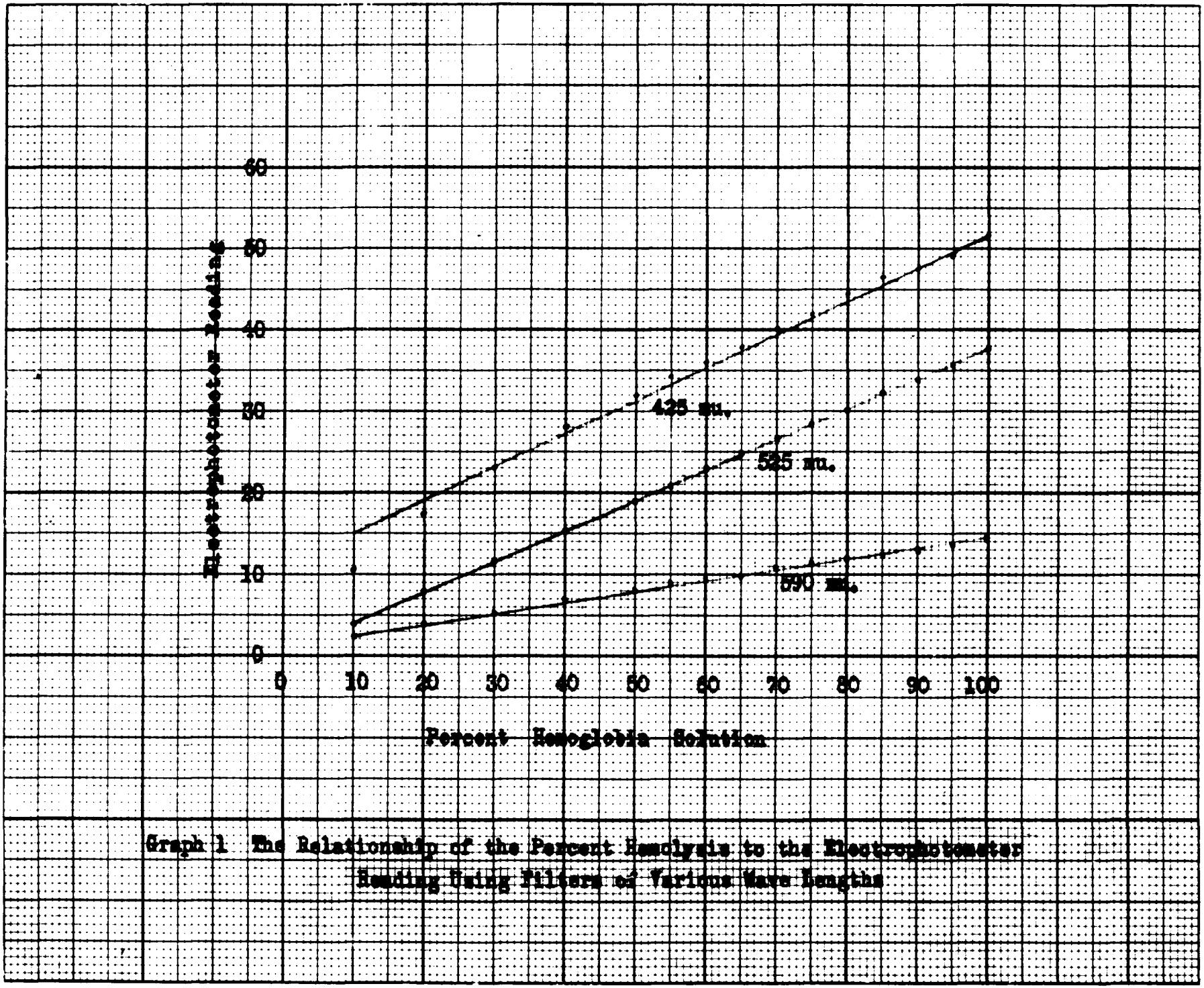
Tube Number		1	2	3	4	5	6	7	8	9	10
Complement ml. (1:100)		.20	.25	.30	.35	.40	.45	.50	.55	.60	.65
Diluent ml.		.80	.75	.70	.65	.60	.55	.50.	.45	.40	.35
Sensitized cells ml.		1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Diluent		Electrophotometer Readings									
Phosphate Buffer	(a)	3	4	6	10	18	20	26	31	39	42
	(b)	2	4	6	11	15	20	24	32	38	43
Ionic Buffer	(a)	24	32	38	46	51	55	59	62	65	69
	(b)	23	32	38	47	52	57	60	63	65	69

Milliliters of undiluted complement necessary for 50 percent hemolysis with:

Phosphate Buffer 0.00575 Ionic Buffer 0.00275

for the electrophotometer readings are plotted in Graph 1. The readings for the 425 millimicron filter did not give a straight line, the values for the lower concentrations dropping considerably below what would be expected. The readings for the 590 millimicron filter covered only 12 divisions on the log scale of the dial, which did not allow the accurate differentiation of readings for concentrations differing only a few percent. The 525 millimicron filter gave a consistent increase in the log scale readings with increased concentration and was selected for use in subsequent determinations. The plotting of these points, as shown in Graph 1, did not coincide exactly with a straight line; however, the variation was later found to be due to differences in the inside diameters of the tubes containing the hemoglobin solutions.

The variation in the tube size was first noticed when repeat determinations on the various concentrations were attempted. To check the reproducability of the readings obtained with the 525 millimicron filter, a hemoglobin solution was prepared by adding 10 milliliters of washed red cells to 400 milliliters of distilled water. The reading for this solution was then adjusted to 40 on the log scale by the addition of distilled water. Using this value to represent 100 percent hemolysis, 25 milliliters of each of the other concentrations (10 to 100 percent) were prepared. Two milliliters of each concentration was pipetted into 15 tubes, making 15 groups of the 15 concentrations. The electrophotometer readings for the tubes containing any given concentration varied considerably. A few tubes were chosen at random, their inside diameters measured, and found to vary in size, with the smallest measuring 0.975 centimeters, and the largest 1.020 centimeters.



Graph 1 The Relationship of the Percent Hemolysis to the Electrophotometer Reading Using Filters of Various Wave Lengths

Since the electrophotometric determinations are based on the amount of light transmittance, the differences in the diameters of the tubes would alter the amount of solution through which the light would have to pass. If the tube were large, the distance through the solution would be increased and the reading lowered, due to the adsorption of more light. If the tube were small, the distance through solution would be decreased and the reading high. To obtain accurate readings with a large number of tubes, the inside diameters of all the tubes must be constant.

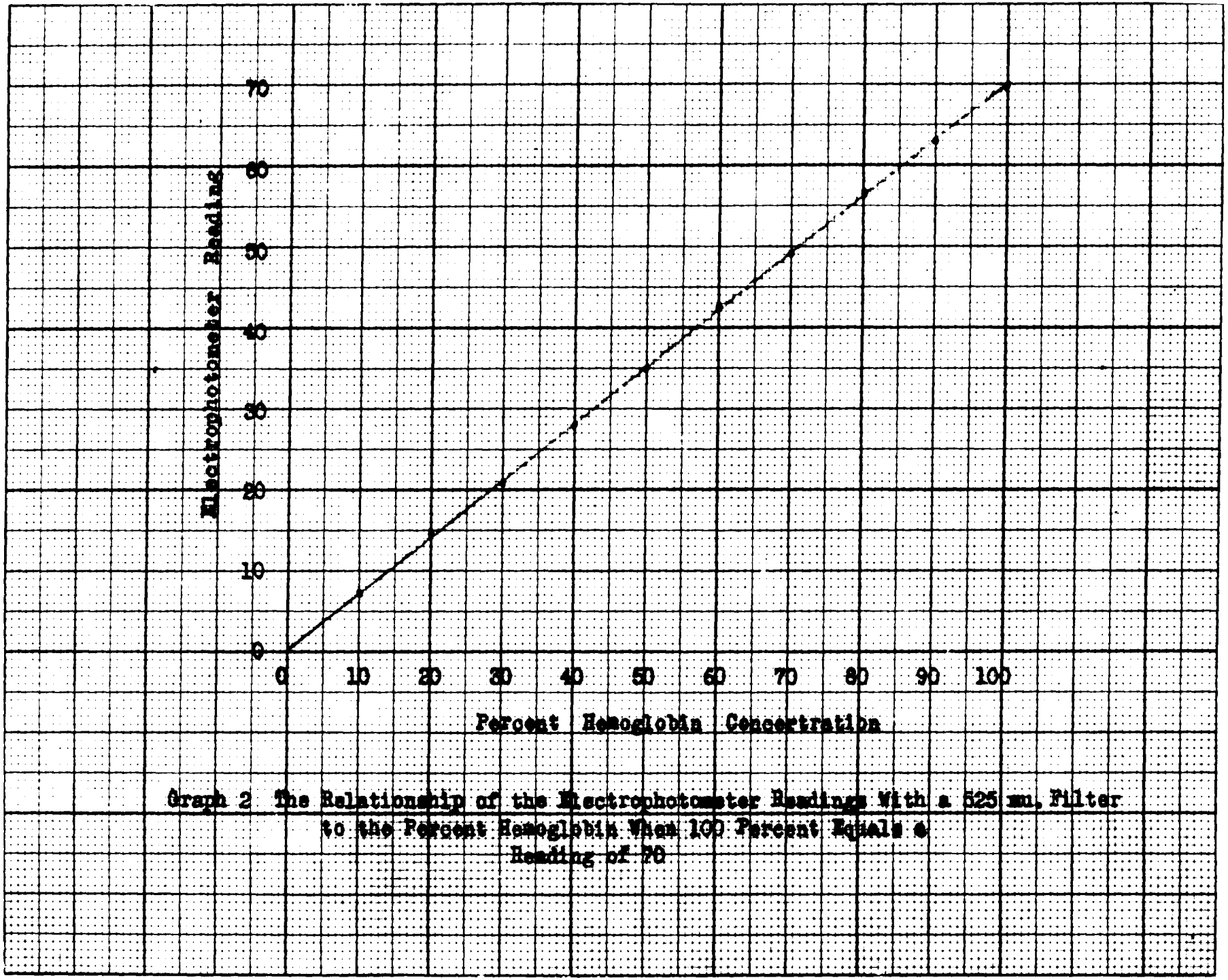
Approximately 3600 tubes were available for use in these tests. They measured three-eighths by three inches and were flanged at the top. The tube size was not the same as the cuvette usually used with this model electrophotometer, and a special cuvette holder was made.

The first step in the standardization of the tube size was the measurement of their inside diameters with calipers. The calipers were set at 0.995 centimeters, and the inside diameter of each tube graded according to the manner in which it fitted over this size measure. The tubes were found to fall into three general classes: small, into which the measure could not be forced; average, which fitted snugly over the measure; and large, which allowed considerable movement after it had been inserted into the tube. A second check on the uniformity of size was then made on the tubes which had been classified as average. This included approximately three-fourths (2700) of the original group of tubes. This second check consisted of preparing 200 milliliters of a hemoglobin solution giving a reading of 40 on the electrophotometer. The solution was pipetted into the tubes in the amount of two milliliters each and the electrophotometer readings taken. Since the solution was standard for

all tubes, those having the same inside diameter should give the same reading. A marked variation was noted in only about 10 percent of the tubes, and these were discarded. The remaining tubes, all giving approximately the same reading, were considered satisfactory and used in subsequent determinations.

f. Red blood cell concentration standardization:--A reading of 40 on the log scale of the drum dial of the electrophotometer was used as the 100 percent hemolysis end point for the determination of the uniformity of the tube size. This value was chosen arbitrarily, since it was approximately in the middle of the scale, where the unit scale size was relatively large and allowed easy determination of slight variations in the readings. When selecting the 100 percent hemolysis value to be used in the tests, however, a larger value was considered more desirable. This allowed a greater range between 0 and 100 percent, thus making the unit interval on the scale representative of less difference in the amount of hemoglobin present in the solution being measured. The unit size on the scale was largest at the 0 end and became smaller as the values increased. Individual scale units were marked off on the dial from 0 to 75, above which the markings were at intervals of five units each. The value 70 was chosen for 100 percent hemolysis, thus making 35 the reading representing 50 percent hemolysis.

Concentrations of hemoglobin, varying from 10 to 100 percent, the same as were used in the testing of the filters, were prepared. The concentration of hemoglobin in this case, however, was adjusted to give a reading of 70 for the 100 percent value. When plotted, the readings for these concentrations gave approximately a straight line as shown in Graph 2.



Graph 2 The Relationship of the Electrophotometer Readings With a 525 mu. Filter to the Percent Hemoglobin When 100 Percent Equals a Reading of 70

The preserved red cells had to be washed before their concentration could be adjusted. The washing procedure was conducted as follows: 10 milliliters of the preserved cells was placed in a graduated centrifuge tube and centrifuged for five minutes at 1000 g. The supernatant fluid, or preservative-blood plasma mixture, was decanted off and approximately five milliliters of phosphate buffer solution added. The tube was then shaken vigorously until all of the cells which had adhered to the bottom of the tube were resuspended. The volume was then made up to 15 milliliters with the phosphate buffer, the tube shaken, and the suspension centrifuged in the same manner as described above. This procedure was repeated until a total of three washings with the phosphate buffer had been completed. Following the last washing, the volume of the packed red cells was measured, a small amount of ionic buffer added, and the cells resuspended by shaking. They were then transferred to a 500 milliliter graduate and ionic buffer added in an amount sufficient to make a 2 percent suspension of the cells. A portion of the buffer was used to rinse the centrifuge tube.

In the tests the red cells were broken down by the action of the complement. For the standardization of the red cell concentration, however, this breakdown had to be brought about in some other way. The addition of distilled water proved satisfactory for this purpose. The ratio of distilled water to the red cell suspension, however, had to be accurate, since the concentration of the resulting hemoglobin solution was critical. This ratio was dependent upon the volume of the red cell suspension and the total volume when all the components were present in a given test, which had been set at two milliliters. Of this amount,

one milliliter was taken up by the antiserum, complement and diluent, the diluent (ionic buffer) being added in amounts sufficient to standardize this volume. The other one milliliter was made up of equal parts of red cell suspension and hemolysin. Therefore, the standardized red cell suspension accounted for 0.5 milliliters of the total volume in any given test. By adding 1.5 milliliters of distilled water to 0.5 milliliters of the red cell suspension, a hemoglobin solution comparable to 100 percent hemolysis in a test was obtained. The readings for hemoglobin solutions prepared in this manner from the 2 percent cell suspension, were uniformly higher than 70, indicating that there was too great a concentration of red cells. To correct for this, the formula $V_2 = \frac{V_1 OD_1}{70}$ was used (Kent et al., 1946). In this relation, V_1 was the volume of the suspension being measured, OD_1 the reading obtained for this suspension, 70 the reading desired, and V_2 the volume needed to give the reading of 70. By inserting the proper values and calculating for V_2, the amount of ionic buffer necessary to add to the suspension to give a reading of 70 was determined. If the reading for the OD_1 value was below 70, the same formula was used to calculate the amount of diluent to be removed. This was accomplished by centrifuging a portion of the suspension and the calculated amount of diluent decanted off. The sedimented cells were then resuspended and returned to the parent suspension. The reading was checked the same as before and, if necessary, additional adjustments made until a value of $70 \pm .5$ was obtained.

An example of the use of the above formula would be as follows. If the reading for the original suspension or OD_1 was 78, and the total volume or V_1 was 140 milliliters, these values would be inserted into the

formula as:

$$V_2 = \frac{140 \times 78}{70}$$

$$V_2 = 2 \times 78$$

$$V_2 = 156$$

The second volume, V_2, which should give a reading of 70, is 156 milliliters or an increase of 16 milliliters over the original volume V_1. Therefore, 16 milliliters of ionic buffer would be added, the suspension thoroughly mixed, and the reading again checked.

g. Selection of complement fixation technique:--In complement fixation tests conducted prior to those reported here, the techniques described by Mayer et al. (1947, 1948b) and Rice (1946, 1947a, b, 1948a) had been used. Both of these techniques utilize the 50 percent hemolytic unit of complement. In the technique described by Mayer, an excess amount of complement was placed with the antigen-antibody system being tested. By titrating the amount of complement present before and after the antigen-antibody complex had acted upon it, and subtracting the latter value from the former, the amount of complement fixed was determined.

In the second technique, that used by Rice, a series of tubes, each containing the same amounts of antigen and antibody, was prepared. Varying amounts of complement, usually 3, 6, 9, and 12 units, were added to these tubes. The degree of reaction was then calculated from the amount of hemolysis occurring in the various tubes.

Both of the techniques mentioned used the slope of the hemolytic curve in the determination of the units of complement which had been fixed in a given reaction. The slope of the line, represented by $\frac{1}{n}$, was the slope of the straight line obtained by plotting the log x which was the

quantity of complement used, against the log $\frac{y}{1-y}$ where y equaled the percentage of hemolysis which had occurred. By using this slope value, the units of complement responsible for any given change in hemolysis could be calculated. If these values were calculated over the entire range of hemolysis obtained, a table could be prepared which would give the amount of complement (in units) responsible for a given amount of hemolysis. These complement values changed, however, with a change of the slope value.

The actual determination of the slope was made by use of von Krogh's alteration equation (von Krogh, 1916):

$$x = K \left(\frac{y}{1-y}\right)^{1/n}$$

or in logarithmic form:

$$\log x = \log K + \frac{1}{n} \log \frac{y}{1-y}$$

In these relations, x represents the volume of complement used and y the corresponding percent of hemolysis. The constants 1/n and K, respectively, denote the slope of the hemolytic curve and the quantity of complement giving 50 percent hemolysis (one unit). The direct calculation of 1/n, however, was made by the use of the method of least squares (Kent et al., 1946) in which the following formula was employed:

$$1/n = \frac{N \cdot \Sigma XY - (\Sigma X \times \Sigma Y)}{N \cdot \Sigma Y^2 - (\Sigma Y)^2}$$

In this relation X was the log x, and Y the log $\frac{y}{1-y}$, and N the number of observations.

The values X and Y had to be calculated for each of the tubes used in a given titration. To facilitate these calculations, tables were

prepared which gave the values for X, Y, and Y^2 over the range of values encountered. In the sample calculation shown in Table II, five values for x (the amount of the complement) were used, since they all gave readings within the range of 20 to 80 percent hemolysis. If the 50 percent end point had been near the smallest, or largest, of the x values, however, only a portion of them could have been used since those at the opposite extreme would have been out of the usable range. A minimum of three values was considered necessary for the calculation, with the 50 percent value falling between them. In other words, if there were only three values within the 20 to 80 percent range, but all were either above or below the 50 percent value, no calculation was made.

The consistency of the slope value was questioned when, upon preliminary calculations using complement titrations, a variation from 0.25 to 0.33 was obtained. A large group of complement titrations, the technique of which will be given later, was conducted, and the slopes calculated. The slope values ranged from 0.23 to 0.38 for the 129 determinations involved. The average for the group was 0.30, which was considerably above the 0.20 given by Mayer et al. (1946b) and Rice (1942) as the optimal value. If the slope were considered to be 0.20 and conversion tables prepared (Rice, 1948a), considerable error would arise when such tables were used in calculating the amount of complement fixed in a test. These slope determinations had been made with values obtained from complement titrations. It was obvious that they did not agree with the generally accepted slope value of 0.20. This discrepancy led to the consideration of the possible effect of either the antigen or antiserum, or both, on this slope value, and tests were conducted to determine what effect they might have.

TABLE II

A SAMPLE SLOPE CALCULATION

x (ml. of complement)	0.30	0.35	0.40	0.45	0.50
X (log of x)	1.477	1.544	1.602	1.653	1.699
Electrophotometer reading	15	22	36	43	48
y (percent hemolysis)	.215	.315	.515	.615	.685
Y (log of y)	1.438	1.663	2.026	2.203	2.337
Y^2	2.068	2.766	4.105	4.853	5.462
XY	2.124	2.568	3.246	3.642	3.970

Values needed in the calculation:

ΣX	ΣY	ΣY^2	ΣXY	$(\Sigma Y)^2$	$(\Sigma X \cdot \Sigma Y)$
7.875	9.667	19.254	15.550	93.451	77.094

Formula used in the calculation:

$$1/n = \frac{(N^* \cdot \Sigma XY) - (\Sigma X \cdot \Sigma Y)}{(N \cdot \Sigma Y^2) - (\Sigma Y)^2}$$

$$= \frac{(5 \cdot 15.550) - (77.094)}{(5 \cdot 19.254) - (93.451}$$

$$= \frac{77.750 - 77.094}{96.270 - 93.451}$$

$$= \frac{.656}{2.891}$$

$$1/n = .23$$

* N is the number of values involved.

The antigen selected for standardization of the technique had been the PR8 strain of human influenza virus Type A. The slope values for titrations made when 0.1 milliliter of the influenza antigen at a dilution of 1:20 was present, differed from those obtained when only complement was present, varying from 0.26 to 0.41 with an average of 0.33 for 123 determinations.

The antiserum selected for these tests had been prepared by the inoculation of rabbits with the influenza virus as is described under the section on antiserum preparation. A series of titrations was made in which 0.1 milliliter of the antiserum was used at a dilution of 1:20, and the slopes calculated. The slope values varied from 0.26 to 0.43 with an average of 0.34 for 105 determinations, which was approximately the same as had been found with antigen present.

The most important determination in a complement fixation test is the titration of the specific reaction between antigen and antiserum. To determine the variability of the slope value when this reaction took place, a series of titrations was made in which the amount of complement was adjusted so that there were sufficient electrophotometric readings in the desired range to allow the calculation of the slope values after a portion of the complement had been fixed by the reaction of influenza antigen and rabbit anti-influenza serum. The slope values in this case ranged from 0.15 to 0.26 for 97 determinations, with an average of 0.21, which agreed closely with the recommended 0.20 value mentioned previously. The variation between individual values, however, was considerably greater than had been desired.

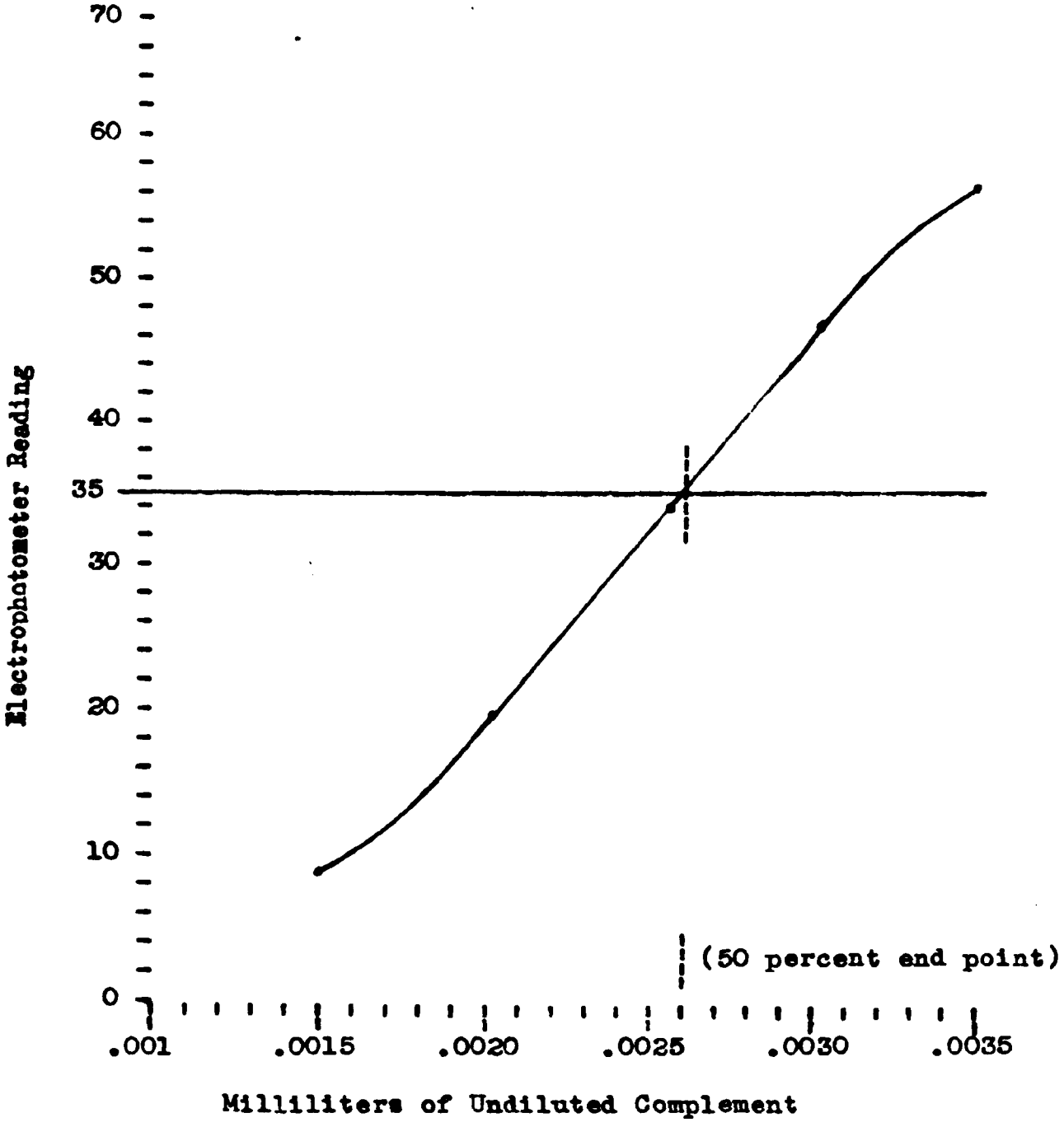
The determination of the amount of reaction occurring in a given titration, and the amount of complement necessary for the production of 50 percent hemolysis, when alone or in the presence of either antigen or antiserum, or both, is dependent upon the slope value in the techniques of both Rice and Mayer. If the slope were considered to be 0.20 for the complement titration, but was actually 0.30 (the average of the slope values obtained for complement titrations), the amount of complement necessary for one unit could not be accurately calculated, and any determinations employing the unit value calculated with the 0.20 slope value would be in error.

The variability of the slope values, when the various reagents were present, and the possible errors which would occur unless separate slope values were used in the preparation of conversion tables for each reaction involved, led to the conclusion that a technique was needed in which the slope calculation was not necessary. In the techniques mentioned previously, the amount of complement necessary for 50 percent hemolysis, or one unit, was determined and a given number of units used in a reaction. Since the amount of complement fixed by a given reaction is used to determine the extent of the reaction, it was considered a more direct approach to determine the actual milliliters of complement involved, and then convert this to units, rather than to determine one unit and work in multiples of this value.

The determination of the exact amount of complement necessary to give 50 percent hemolysis, either in a complement titration or when one or more reagents were present, was possible by plotting the electrophotometer readings against the milliliters of complement used. Where the

curve crossed the line representative of 50 percent hemolysis (reading of 35), it would also intersect the coordinate representing the amount of complement necessary to produce this degree of hemolysis. The curve shown in Graph 3 was obtained from a titration of complement and was carried out as described in the section on complement titration. The same type of graph can be prepared for the readings obtained for any titration, or reaction measurement, as long as the amount of hemolysis gives readings which are within the range of 20 to 80 percent and are distributed to both sides of the 50 percent hemolysis value.

When a large number of determinations was to be made, the plotting of the respective values became a task of considerable magnitude. As will be noted in Graph 3, only two of the readings are actually used for the determination of the 50 percent end point. These are the ones just above and just below the reading of 35 (50 percent hemolysis). To eliminate the necessity of plotting the electrophotometer readings on graph paper, connecting the points and determining the milliliters of complement for each titration, a table was prepared which gave the proportionate distance, between any two complement values, where the curve would cross the line representative of 50 percent hemolysis. This table was prepared by using a large sheet of graph paper and making two columns of values from 0 to 70 as shown in Figure 3. By extending a straight edge from the value 1 of the left column to all of the values greater than 35 in the right column, then from 2 to all of the values over 35, and so on through 34, the proportionate distance between these columns (considering the total distance to be 10 unit spaces) where the curve intersected the line of 50 percent hemolysis (35) was determined and recorded. For



Graph 3 The Relationship of the Electrophotometer Readings to the Amount of Complement

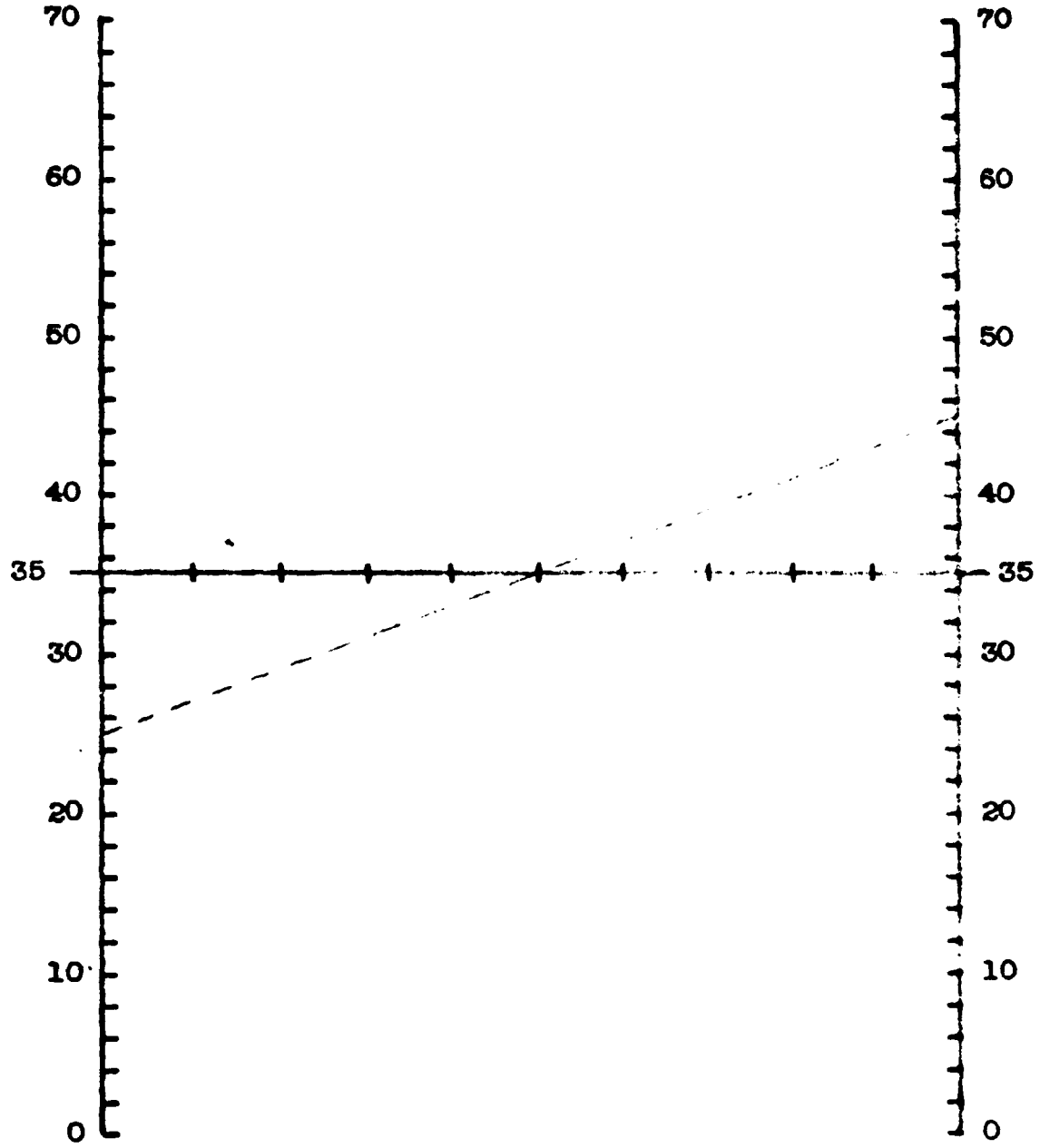


Figure 3 Chart Used for Determination of Proportionality Values

example, if the reading immediately below the 50 percent value was 25 and the one above was 45, a line connecting the two would intersect the 50 percent line half-way between the columns, as shown in Figure 3, and give a proportionate value of 5. By arranging these values as shown in Table III, the proportionate distance where a line, between any two of the readings for different amounts of complement would cross the 50 percent hemolysis line, could be determined. This eliminated the necessity of plotting the readings and could be used with any amounts of complement employed.

The use of the table for the determination of the proportionate distance where the curve crosses the 50 percent hemolysis value not only eliminated the necessity of plotting the electrophotometer readings, but also reduced the number of readings which had to be made. Only two readings were necessary to determine the amount of reaction occurring in a given titration, those being the ones on either side and closest to the 50 percent hemolysis value. The use of this procedure eliminated the necessity for the use of a slope value in any of the determinations and avoided the inherent errors which would have resulted from the use of such a value.

h. Calculation of data:--The use of a large number of different antigens and antiserums made it necessary to adopt a method for the calculation of results which would facilitate the easy identification of the various reagents. As will be shown later, symbols for the individual reagents were used, but for the calculation of the amounts of complement fixed, either in terms of milliliters of undiluted complement or units, additional symbols were needed. The notations suggested by Thompson et

TABLE OF PROPORTIONATE VALUES

	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70
1	10	10	9	9	9	8	8	8	8	8	8	7	7	7	7	7	7	7	7	6	6	6	6	6	6	6	6	6	5	5	5	5	5	5	5
2	10	9	9	9	8	8	8	8	8	8	7	7	7	7	7	7	7	6	6	6	6	6	6	6	6	6	6	5	5	5	5	5	5	5	5
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6	10	9	9	9	8	8	8	8	8	7	7	7	7	7	6	6	6	6	6	6	6	6	6	5	5	5	5	5	5	5	5	5	5	4	4
7	10	9	9	9	8	8	8	8	8	7	7	7	7	7	6	6	6	6	6	6	6	6	5	5	5	5	5	5	5	5	5	4	4	4	4
8	10	9	9	9	8	8	8	8	8	7	7	7	7	6	6	6	6	6	6	6	6	6	5	5	5	5	5	5	5	5	4	4	4	4	4
9	10	9	9	9	8	8	8	8	8	7	7	7	7	6	6	6	6	6	6	6	5	5	5	5	5	5	5	5	5	4	4	4	4	4	4
10	10	9	9	9	8	8	8	8	8	7	7	7	6	6	6	6	6	6	6	6	5	5	5	5	5	5	5	5	4	4	4	4	4	4	4
11	9	9	9	9	8	8	8	7	7	7	7	7	6	6	6	6	6	6	6	5	5	5	5	5	5	5	5	4	4	4	4	4	4	4	4
12	9	9	9	9	8	8	8	7	7	7	7	6	6	6	6	6	6	6	5	5	5	5	5	5	5	5	5	4	4	4	4	4	4	4	4
13	9	9	9	8	8	8	8	7	7	7	7	6	6	6	6	6	6	6	5	5	5	5	5	5	5	4	4	4	4	4	4	4	4	4	4
14	9	9	9	8	8	8	8	7	7	7	6	6	6	6	6	6	6	5	5	5	5	5	5	5	4	4	4	4	4	4	4	4	4	4	4
15	9	9	9	8	8	8	8	7	7	7	6	6	6	6	6	5	5	5	5	5	5	5	5	4	4	4	4	4	4	4	4	4	4	4	4
16	9	9	9	8	8	8	7	7	7	6	6	6	6	6	6	5	5	5	5	5	5	5	4	4	4	4	4	4	4	4	4	4	4	4	4
17	9	9	8	8	8	7	7	7	7	6	6	6	6	6	6	5	5	5	5	5	5	4	4	4	4	4	4	4	4	4	4	4	4	4	3
18	9	9	8	8	8	7	7	7	6	6	6	6	6	6	5	5	5	5	5	4	4	4	4	4	4	4	4	4	4	4	4	3	3	3	3
19	9	9	8	8	8	7	7	7	6	6	6	6	6	5	5	5	5	5	4	4	4	4	4	4	4	4	4	4	4	4	3	3	3	3	3
20	9	9	8	8	8	7	7	7	6	6	6	6	6	5	5	5	5	5	4	4	4	4	4	4	4	4	4	4	4	4	3	3	3	3	3
21	9	9	8	8	7	7	7	6	6	6	6	5	5	5	5	5	4	4	4	4	4	4	4	4	4	3	3	3	3	3	3	3	3	3	3
22	9	9	8	8	7	7	6	6	6	6	5	5	5	5	5	4	4	4	4	4	4	4	4	3	3	3	3	3	3	3	3	3	3	3	3
23	9	9	8	8	7	7	6	6	6	5	5	5	5	5	4	4	4	4	4	4	4	3	3	3	3	3	3	3	3	3	3	3	3	3	2
24	9	8	8	7	7	7	6	6	6	5	5	5	5	4	4	4	4	4	4	3	3	3	3	3	3	3	3	3	3	3	3	3	3	2	2
25	9	8	8	7	7	6	6	6	5	5	5	4	4	4	4	4	4	3	3	3	3	3	3	3	3	3	3	2	2	2	2	2	2	2	2
26	9	8	7	7	6	6	6	5	5	5	4	4	4	4	4	3	3	3	3	3	3	3	3	3	2	2	2	2	2	2	2	2	2	2	2
27	9	8	7	7	6	6	5	5	5	4	4	4	4	4	4	3	3	3	3	3	3	3	2	2	2	2	2	2	2	2	2	2	2	2	2
28	9	8	7	6	6	5	5	5	4	4	4	4	3	3	3	3	3	3	3	3	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
29	8	8	7	6	6	5	5	4	4	4	4	3	3	3	3	3	3	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1
30	8	7	6	5	5	4	4	4	4	3	3	3	3	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1
31	8	7	6	5	4	4	4	3	3	3	3	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	1
32	7	6	5	4	4	3	3	3	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
33	6	5	4	4	3	3	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
34	5	4	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0

al. (1949) were used for this purpose. The origin of these symbols was outlined by him and will not be repeated. A list of those used in the calculation of the results given here are as follows:

(1) K = the amount of complement necessary to produce 50 percent hemolysis.

(2) $K_\bullet$ = K when serum, antibody, and antigen are absent such as in the reaction called "complement titration." This value is the arbitrary unit, making $K_\bullet = 1$.

(3) K_s = K when serum alone is present, and

(4) K_a = K when antigen alone is present.

(5) $K_{s,a}$ = K when both serum and antigen are present.

All of these notations refer to the values when expressed in terms of units of complement. The same notation was used when the values were in terms of milliliters of undiluted complement, except that the letter K was primed. Therefore, when expressed in terms of milliliters of undiluted complement, the symbols were K'_s, K'_a, and $K'_{s,a}$. If specific serums or antigens were involved, the symbols representative of them were inserted in place of the "s" or "a". This made it possible to use more than one serum or antigen in a group of tests and maintain its identity.

The use of these symbols in the indirect test calculations necessitated the introduction of an additional notation representing the reaction where both chicken and guinea pig serums were present. This was designated as:

(6) $K_{s,s,a}$ (units) or $K'_{s,s,a}$ (milliliters of undiluted complement)

which, when subtracted from the values obtained for (5), gave the titer of the chicken serum.

A clearer understanding of the method of calculation will be possible if the values for a typical titration are used, and a detailed calculation carried out.

The titer of the complement was determined first. The K_e value is always 1; therefore, only the K'_e, which is in terms of milliliters of undiluted complement, can be used since, as will be shown, the other K' values must be divided by it to determine the K, or unit, values. In this case, the K'_e was 0.0032.

The values for the K'_a and K'_s were calculated next by use of the proportionality table (Table III) and were the tests on the anticomplementary activity of these reagents. The symbols for the actual reagents were substituted for the "a" and "s", respectively. The calculation for the influenza antigen (FA) was: $K_{FA} = \frac{K'_{FA}}{K'_e} - 1$. The subtraction of 1 accounts for the one unit necessary to produce the 50 percent hemolysis which was measured. When the actual values were inserted, the actual calculation was: $K_{FA} = \frac{.0046}{.0032} - 1 = 1.4 - 1 = 0.4$ units. The anticomplementary values for the guinea pig anti-influenza serum (FGPS) and chicken anti-influenza serum (FCS) were calculated in the same manner.

$$K_{FGPS} = \frac{.0044}{.0032} - 1 = 1.3 - 1 = 0.3 \text{ units}$$

$$K_{FCS} = \frac{.0050}{.0032} - 1 = 1.5 - 1 = 0.5 \text{ units}$$

These values represented the anticomplementary activity of the individual reagents, and had to be taken into consideration in the calculation of the amount of fixation occurring in any reaction in which they were involved.

The next calculations to consider were the control reactions involving all possible combinations of reagents normally considered to have no

effect on the complement. When a specific reaction was being measured, and there was a number of reagents present, control tests had to be made using all combinations of all reagents. This was done to eliminate the possibility that a second reaction was taking place which would have some effect on the complement. The first of these combinations was the influenza antigen and chicken anti-influenza serum. The antibodies from the chicken, although capable of combining with the antigen, should not produce a complex which would affect the complement, and this test was made as a check on that assumption. The anticomplementary values for these two reagents had to be taken into consideration and the calculation made as follows:

$$K_{FA + FCS} = \left(\frac{K'_{FA + FCS}}{K'_{\bullet}} - 1 \right) - (K_{FA} + K_{FCS})$$

$$= \left(\frac{.0050}{.0032} - 1 \right) - (0.4 + 0.5)$$

$$= (1.6 - 1) - 0.9$$

$$= 0.6 - 0.9$$

$$K_{FA + FCS} = - 0.3 \text{ units}$$

The second control test involved the guinea pig anti-influenza serum (FGPS) and the chicken anti-influenza serum (FCS). This was an important control since, if the material inoculated into the guinea pig to produce the influenza antiserum had contained chicken material of a nonspecific type, the antibodies formed might react with a similar substance in the chicken serum. The calculation for this test was:

$$K_{FGPS + FCS} = \left(\frac{K'_{FGPS + FCS}}{K'_{\bullet}} - 1\right) - (K_{FGPS} + K_{FCS})$$

$$= \left(\frac{.0056}{.0032} - 1\right) - (0.3 + 0.5)$$

$$= (1.8 - 1) - 0.8$$

$$= 0.8 - 0.8$$

$$K_{FGPS + FCS} = 0.0$$

The direct test involving the influenza antigen (FA) and guinea pig anti-influenza serum (FGPS) was then calculated in the same manner as the two previous reactions. When a large number of tests was being made by the indirect technique, the direct test had to be standardized and was actually an indicator system, since all indirect tests contained a complete direct test system plus an additional antiserum (chicken) which was the material being tested.

The calculations for the direct reaction were as follows:

$$K_{FA + FGPS} = \left(\frac{K'_{FA + FGPS}}{K'_{\bullet}} - 1\right) - (K_{FA} + K_{FGPS})$$

$$= \left(\frac{.0580}{.0032} - 1\right) - (0.4 + 0.3)$$

$$= (18.1 - 1) - 0.7$$

$$= 17.1 - 0.7$$

$$K_{FA + FGPS} = 16.4$$

The indirect test was calculated next. Both the guinea pig and chicken anti-influenza serums were involved in this test and as will be noted, the anticomplementary values for each are taken into consideration.

$$K_{FA + FGPS + FCS} = \left(\frac{K'_{FA+FGPS+FCS}}{K'_{\bullet}} - 1\right) - (K_{FA}+K_{FGPS}+K_{FCS})$$

$$= \left(\frac{.0220}{.0032} - 1\right) - (0.4 + 0.3 + 0.5)$$

$$= (6.9 - 1) - 1.2$$

$$= 5.9 - 1.2$$

$$K_{FA + FGPS + FCS} = 4.7 \text{ units}$$

The difference in the number of units of complement fixed in the two tests indicated the extent of binding of the antigen by the chicken antiserum. When the units fixed in the indirect test were subtracted from the number obtained for the direct test, this binding by the chicken antiserum was determined in terms of units of complement. This value was the indirect titer of the chicken serum and designated by the symbol I_{cs}. In terms of the notations it was equivalent to:

$$I_{cs} = K_{FA + FGPS} - K_{FA + FGPS + FCS}$$

which, when calculated with the actual values, was:

$$I_{cs} = 16.4 - 4.7$$

$$I_{cs} = 11.7 \text{ units}$$

IV. TITRATION OF REAGENTS

a. Complement titration:--The amount of fixation was measured in the tests in terms of milliliters of complement. It was desirable, however, for ease of interpretation, to convert these amounts to units. Therefore, the determination of the amount of complement necessary to produce 50 percent hemolysis, or one unit, was necessary.

The complement, in the form of frozen guinea pig serum, was removed from storage, thawed under flowing tap water, and diluted 1:100 with ionic buffer.

A suspension of sheep red blood cells was standardized electrophetometrically and mixed with an equal volume of hemolysin. The cells were thus made sensitive to the action of complement. By mixing varying amounts of complement with these sensitized cells, the exact amount necessary to produce 50 percent hemolysis, which would be one unit, could be determined. A series of reactions in which varying amounts of complement were employed, constituted a complement titration and was prepared as shown in Table IV. When the proportionality table (Table III) was used to determine the proportionate distance between the amounts giving the readings of 34 and 48 in the titration shown, a value of 1 was obtained. Therefore, since in this case the interval between the volumes of complement was 0.05, the proportionate amount was 0.1 of this or 0.005, making 0.255 milliliters of the 1:100 dilution of complement necessary for the 50 percent hemolysis. This was rounded off to 0.26 or 0.0026 milliliters of undiluted complement.

TABLE IV

COMPLEMENT TITRATION

Reagent	Tube Number 1	2	3	4	5
Complement 1:100 (ml.)	0.15	0.20	0.25	0.30	0.35
Ionic Buffer (ml.)	0.85	0.80	0.75	0.70	0.65
Sensitized Red Cells (ml.)	1.0	1.0	1.0	1.0	1.0
Electrophotometer Reading	9	20	34	48	57

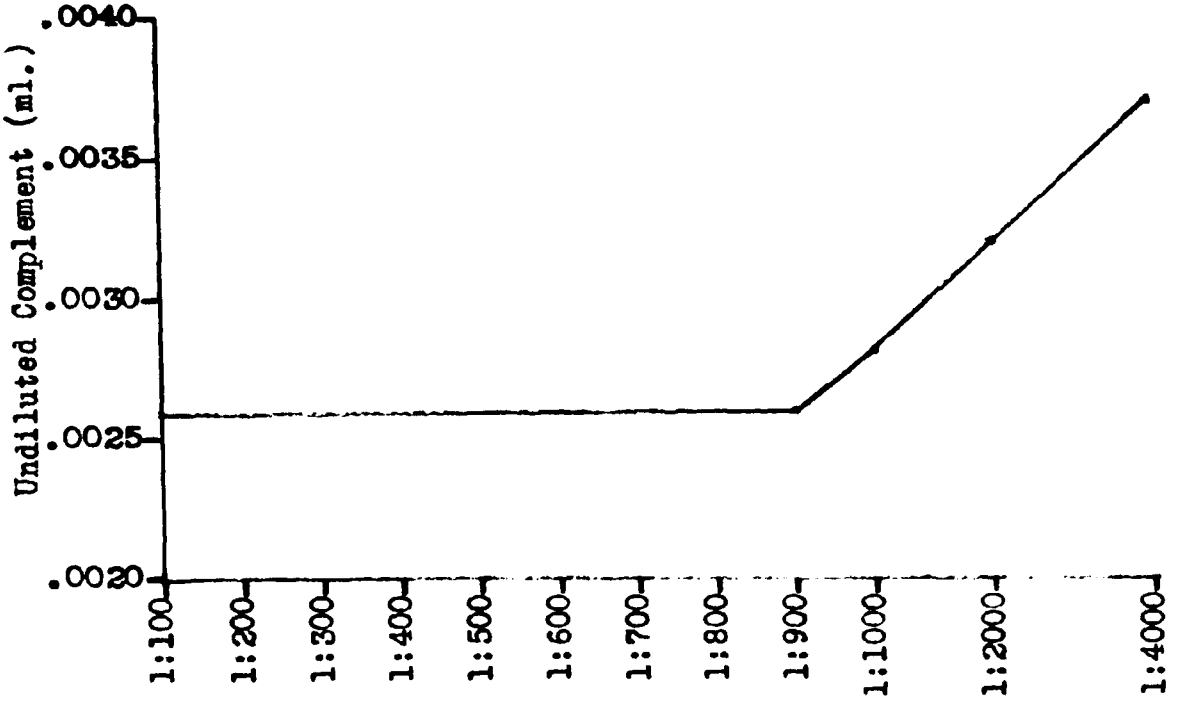
b. Hemolysin titration:--The technique described by Kent (1946) was used to determine the optimal concentration of hemolysin. This has been described by Wadsworth (1947) as the concentration beyond which further increase fails to enhance appreciably the hemolytic activity of complement. Therefore, if varying concentrations of hemolysin were added to a series of tubes, each containing 1 unit of complement, all tubes containing 1 or more units of hemolysin would show 50 percent hemolysis, since there would be sufficient hemolysin present to allow all of the complement activity to be expressed. However, in those tubes containing less than 1 unit of hemolysin, the complement activity would be lessened and the amount of hemolysis would fall below 50 percent. In other words, as the amount of hemolysin decreases below a certain magnitude, the amount of complement necessary to produce 50 percent hemolysis increases.

The hemolysin dilutions were prepared over a range of from 1:100 to 1:4000. Ten milliliters of each of the dilutions was mixed with an equal volume of standardized red cells and allowed to stand at room temperature for at least 10 minutes (Mayer et al., 1946b). Twelve titrations of the type shown in Table V were then prepared. These were merely complement titrations with varying amounts of hemolysin by which the effect of the hemolysin concentration could be determined. The titrations were conducted in the same manner as described for the complement titration, but the red blood cells were, in each case, sensitized with a different concentration of hemolysin. The effect of the change in the hemolysin concentration on the complement unit was best seen when the amount of complement necessary for 50 percent hemolysis was plotted against the concentrations of hemolysin, as shown in Graph 4. The curve shows that

TABLE V

HEMOLYSIN TITRATION

Hemolysin dilution	Complement 1:100 (ml.)					Complement Unit
	.20	.25	.30	.35	.40	
	Electrophotometer Reading					
1:100	-	34	48	-	-	0.0026
1:200	-	32	45	-	-	0.0026
1:300	-	31	50	-	-	0.0026
1:400	-	32	51	-	-	0.0026
1:500	-	30	49	-	-	0.0026
1:600	-	29	47	-	-	0.0026
1:700	-	32	48	-	-	0.0026
1:800	-	31	51	-	-	0.0026
1:900	-	28	48	-	-	0.0026
1:1000	-	24	44	-	-	0.0028
1:2000	-	-	31	42	-	0.0032
1:4000	-	-	-	27	49	0.0037



Graph 4 Graphical Determination of Hemolysin Titer

there was no change in the amount of complement necessary to produce 50 percent hemolysis at the hemolysin dilutions of 1:900 or less; however, above this dilution, larger amounts of complement were necessary. The titer, therefore, was considered to be 1:900, or the concentration which, when mixed with an equal volume of standardized sheep red blood cells, contained one unit of hemolysin.

To insure the presence of sufficient hemolysin, a dilution of 1:500 was used in all tests. This dilution contained approximately 2 units as recommended by Wadsworth (1939). The titer of the hemolysin was checked every two to three weeks and varied within the range of 1:800 to 1:1000. The dilution used in the tests was maintained at 1:500, however, since the 2-unit value was approximate and used only as a safeguard against a deficiency of this reagent.

V. PREPARATION OF TEST REAGENTS

A. Antigen

The antigens used in the studies reported here were Human Influenza virus (PR8 strain) Type A, normal chicken tissue extract, and extracts of lymphoid liver tumors and pectoral muscle tumors from chickens showing visceral lymphomatosis. The Influenza virus was used for the standardization of the test technique. The normal tissue extract served as a control on the normal tissue reactions of the tumor materials, and the tumor extracts were the test antigens used in attempts to determine the presence of the tumor agent.

a. Influenza virus:--The virus* had been cultivated in the allantoic fluid of chick embryos, harvested when the embryos were 12 to 14 days of age, and formalin added in an amount sufficient to make a 0.2 percent solution. This inactivated the virus and served as a preservative. When treated in this manner, the virus suspension remained apparently unaltered for an indefinite period, if stored at 2-4° C. It was given the name "Flu Antigen" and designated by the symbol FA.

b. Normal tissue antigen:--An extract of normal chicken tissue was prepared for use as a control on the normal tissue reaction which might occur when the tumor materials were used in the tests. The principal tumor antigens, as will be described later, were prepared from tumorous livers. The control antigen, therefore, needed to contain as nearly as possible, the same tissue components, with the objective of making the

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* Obtained from Lederle Laboratories Division of the American Cyanamid Company, Pearl River, New York.

only difference between the two materials the presence of the agent in the tumor antigen.

The liver, spleen and thymus were selected as the tissues which would furnish the materials necessary for the production of a satisfactory control normal tissue antigen. The liver supplied normal liver cell components, while the spleen and thymus, due to their relatively high lymphoid cell content, supplied components of this type of cell. The lymphoid cells of the spleen and thymus were not identical to those found in the lymphoid tumors, but were considered to be the best available. The tissues were secured from frozen materials that had been stored in the dry ice chest at -70° C. They had been removed from chickens which were the progeny of a single mating, designated as mating number D818. These birds had been raised in complete isolation, having been hatched and reared in an isolated pen within a quarantined building. They were approximately 2100 days of age when killed, and had shown no evidence of lymphoid tumors or other disease conditions at any time.

For the actual preparation of the normal tissue antigen, samples of liver, spleen and thymus tissue were removed from storage and thawed under flowing tap water. The tubes were then opened and the tissues placed in the bowl of a Waring blendor in the proportions of approximately 20 grams of liver, 2 grams of thymus, and 5 grams of spleen. Two hundred and fifty milliliters of sterile phosphate buffer was added, and the mixture blended for five minutes. The resultant homogenate was then transferred to sterile lusteroid centrifuge tubes and spun in the multispeed attachment of a refrigerated International Number 2 centrifuge for 10 minutes at 34,000 g. Three distinct layers were formed in the tubes,

the bottom layer consisting of particulate material, a middle layer of very small particles and soluble materials, and a top layer of fatty materials. The middle layer was removed by carefully siphoning it from between the other two layers. The top and bottom portions were then discarded and the material which had been siphoned off transferred to additional sterile lusteroid tubes. The second group of tubes, containing the original middle layer, was centrifuged for 10 minutes at 4600 g and the central portion siphoned off the same as had been done with the first centrifugate. It was then diluted with phosphate buffer until a volume of 250 milliliters was obtained, and transferred to serum vials in 1, 5, and 10 milliliter amounts. The vials were sealed with a flame, and frozen by placing them directly into a mechanical freezer at -35° C. This material, the normal tissue antigen, was designated as NTA, N representing normal, T-tissue and A-antigen.

c. Tumor antigen:--The chickens used as a source of tumorous material had been, with two exceptions, inoculated with filtrates of the RPL strain 12 tumor when they were one day of age. All of the methods of preparation were designed to furnish a suspension of the tumor agent which could be used to stimulate antibody production and serve as an antigen in the complement fixation reactions.

d. Tumor antigen-L1:--Five chickens served as a source of the tumorous livers and serums used for the preparation of this antigen. When, on physical examination, it was evident that the livers were enlarged, each bird was bled by cardiac puncture, the serum recovered, and stored in a dry ice chest. The bird was then killed by electrocution and opened aseptically. A portion of the liver was removed, minced with scissors

and transferred to pyrex test tubes. These were sealed with a flame and stored in the dry ice chest.

At the time of preparation of this antigen, which was designated as TA-L1 (tumor antigen - liver 1), the tumorous material, totaling 25 grams, was removed from the freezer, thawed under running tap water, placed in a Waring blendor with 100 milliliters of phosphate buffer and blended for five minutes. While still in the blendor, 81 milliliters of serum from the same birds was added. The mixture was blended for another minute, transferred to lusteroid centrifuge tubes, and spun in the multispeed attachment of a refrigerated International Number 2 centrifuge at 34,000 g for 10 minutes. The supernatant fluid was siphoned off, placed in glass serum tubes which were sealed, and frozen slowly in an alcohol-dry ice bath. The temperature of the bath was first adjusted to 0° C, the tubes placed in it and the temperature lowered one degree a minute until it reached -30°, at which time the tubes were transferred to a dry ice chest.

e. Tumor antigen-L2:--In this preparation, an attempt was made to concentrate the tumor agent and at the same time, decrease as much as possible the tissue component content. Fifty-two grams of tumorous liver material was placed in a Waring blendor with approximately nine volumes of cold phosphate buffer (450 milliliters), and blended for five minutes. It was then transferred to large glass centrifuge tubes and spun for 20 minutes at 5,000 g in the angle head of a refrigerated International Number 2 centrifuge. The supernatant fluid, which was quite turbid, was siphoned off into a flask immersed in ice water, and the sediment, consisting of the larger particulate materials, was discarded. A portion

of the supernatant fluid was then transferred to six lustereid tubes having a capacity of approximately 15 milliliters. These six tubes, which were the capacity of the multispeed attachment head, were spun for 120 minutes at 30,000 g. The supernatant fluid was poured off, the tubes allowed to drain, and then refilled with an additional 15 milliliters of the original material. This was repeated until a total of four volumes of approximately 15 milliliters each, had been centrifuged in each tube. The pellets increased in size with each centrifugation, and after the fourth run, each contained the sedimented materials from approximately 60 milliliters of the original material. Each pellet was resuspended in 10 milliliters of phosphate buffer, returned to the multispeed attachment, and spun for five minutes at 9,000 g. The supernatant fluid was then siphoned off, and the sediment of each tube re-extracted with a second 10 milliliters of phosphate buffer. The supernatant fluid from the second extraction was added to that obtained from the first extraction and the mixture transferred to serum tubes. These were frozen with the aid of an alcohol-dry ice bath, as previously described, and stored in the dry ice chest.

f. Tumor antigen-L3:--A third tumor antigen, TA-L3, was prepared from 150 grams of tumorous liver tissue. Two thousand milliliters of phosphate buffer was added to the tumor material, and the mixture blended for five minutes in the Waring blendor. The blendor capacity was limited to approximately 700 milliliters, so the tumor material was divided into three parts and each portion blended with approximately one-third of the buffer solution. The three batches were then mixed in a large flask and 96 grams of Celite Number 503 added. This was mixed for five minutes

with a sterile stirring rod, and filtered through number 1 Whatman filter paper and then passed through the continuous flow bowl of a steam-driven Sharples centrifuge at 70,000 g, with the rate of flow adjusted to approximately 2,000 milliliters per hour. After all the material had passed through the centrifuge bowl, the system was washed with 300 milliliters of cold buffer solution. The centrifuge was then stopped, 20 milliliters of sterile glass beads added, the inlet and outlet openings plugged, and the bowl shaken vigorously to free the sediment which had accumulated on the sides of the bowl. The resulting suspension was transferred to large centrifuge tubes and an additional 100 milliliters of buffer placed in the bowl to remove any sediment still adhering to the walls or the glass beads. This was added to the material in the centrifuge tubes, which were spun in the angle head of a refrigerated centrifuge for 20 minutes at 9,000 g. The supernatant fluid was siphoned off and the sediment resuspended in 50 milliliters of phosphate buffer. The tubes were then recentrifuged and the supernatant fluid mixed with that recovered from the previous spin. After thorough mixing, the material was placed in a batch bowl of the Sharples centrifuge and spun for 10 minutes at 10,000 g. The supernatant fluid from this centrifugation constituted the tumor antigen-L3 which was placed in serum tubes, sealed, frozen and stored as described for the L2 antigen.

g. Infected chicken serum:--The serums of birds having visceral tumors, which had been induced by the inoculation of RPL strain 12 filtrates, were used as antigens. Fifty-four serum tubes containing this type of serum were removed from storage, thawed under flowing tap water, pooled, and centrifuged at 5,000 g for 20 minutes. The centrifugation removed

precipitates which formed in some of the samples when they were thawed. The supernatant fluid was siphoned off and placed in serum tubes which were sealed, frozen in an alcohol-dry ice bath and stored in the dry ice chest. This infected chicken serum, which totaled 920 milliliters after the centrifugation, was designated as ICS and was used as an antigen in various tests and for the inoculation of animals for the production of antiserums.

h. Normal chicken serum:--The serums of randomly selected chickens can not be considered as free of the agent of visceral lymphomatosis. Normal appearing birds can apparently transmit the infection through the egg to their offspring, indicating the presence of the agent in the hen producing these eggs (Cottral, 1949). Since the occurrence of tumors in infected birds reaches a peak at around 200 to 250 days of age (Waters, 1947), a group of birds which had lived considerably longer without showing infection, would be a more likely source of agent-free material. A group of birds from one of the susceptible lines of chickens (Line 15) at the Regional Poultry Research Laboratory had been reared in complete isolation, and at the age of 2100 days, had shown no evidence of infection. These were the D818 group which had furnished the tissues for the normal tissue antigen. Serum had been secured from these birds at the time they were sacrificed and was used as a source of normal chicken serum. This served as a control on the infected chicken serum, when used as an antigen and in the inoculations for the production of antiserums.

In order to differentiate this normal chicken serum from the serums taken from birds, which were later used for inoculations, the symbol NCSA was used.

A group of 11 tumor antigens was prepared specifically for the inoculation of chickens in an attempt to produce a material that might stimulate the production of antibodies which would be detectable by the indirect complement fixation technique. These antigens were prepared from strain 12 filtrate-induced tumors with the exception of one which employed strain 12 pectoral muscle tumors and a second prepared from naturally occurring liver tumors. The pectoral tumor had been induced by the transplantation of viable tumor cells rather than a filtrate of the agent (Burmester et al., 1946).

i. Tumor antigen-PT4:--The pectoral muscle tumor antigen, designated TA-PT4 was prepared by mincing 15 grams of pectoral tumor as described by Olsen (1940) and suspending the mince in 135 milliliters of 0.85 percent saline. This was stirred vigorously and filtered through two layers of fine gauze. The filtrate, totaling 112 milliliters, was transferred to a large centrifuge tube and 2.25 milliliters of a 10 percent solution of formaldehyde added. This was mixed thoroughly and allowed to stand in the refrigerator for seven days at 4° C. On the seventh day the material was placed in serum vials in 10 milliliter volumes, sealed, and stored at -35° C.

j. Tumor antigen-L4:--The TA-L4 was prepared in the same manner as the TA-PT4. A 40-gram sample of liver tumor was minced and suspended in 360 milliliters of saline which was filtered through gauze. The filtrate was divided into three portions of 100 milliliters each and 10 percent formaldehyde added in amounts sufficient to make concentrations of 0.05 percent, 0.20 percent, and 0.80 percent. These were allowed to stand in the refrigerator for one week, and then transferred to serum tubes and

stored. The three materials were identified according to the concentration of the formaldehyde present, as:

TA-L4A - 0.05 percent formaldehyde

TA-L4B - 0.20 percent formaldehyde

TA-L4C - 0.80 percent formaldehyde

k. Tumor antigen-L5:--The TA-L5 was prepared in the same manner as described for TA-L3 except that the procedure was stopped after the washing with 300 milliliters of phosphate buffer. The sediment remaining after the washing was suspended in approximately 200 milliliters of the buffer and divided into three equal parts. Formaldehyde was then added as described for the TA-L4, and the resulting antigens designated as TA-L5A, TA-L5B, and TA-L5C.

l. Tumor antigen-L7:--The next antigen, TA-L7 was prepared from 140 grams of liver tumor which was blended in a Waring blender for three minutes with 500 milliliters of phosphate buffer, and then mixed with an additional 2100 milliliters of the buffer. This was filtered through three layers of gauze and the filtrate passed through the continuous flow bowl of a Sharples centrifuge at 30,000 g with a flow rate of 2,000 milliliters per hour. The sediment was discarded and the supernatant fluid passed through the centrifuge again, this time at 60,000 g with the same flow rate. The supernatant fluid from this centrifugation was discarded and the sediment suspended in 100 milliliters of phosphate buffer. A clarifying spin of 20 minutes at 2,000 g was carried out in the refrigerated centrifuge. The supernatant fluid was then decanted off and divided into two parts. The first portion, designated as TA-L7A was placed in serum tubes, sealed and stored. Formaldehyde solution (10

percent) was added to the second part in an amount sufficient to make a 0.05 percent concentration and was designated as TA-L7B.

m. Tumor antigen-L9:--This antigen was prepared from a portion of the same pool of tumor used for TA-L7. A 100-gram sample was mixed with 400 milliliters of 0.85 percent saline and blended for five minutes in a Waring blender. This was transferred to large centrifuge tubes, and spun in the refrigerated centrifuge at 4,000 g for 20 minutes. The supernatant fluid was then decanted off and allowed to stand in the refrigerator overnight at 4° C. It was then recentrifuged at 4,000 g for 20 minutes and the supernatant fluid, which constituted the TA-L9, decanted off and stored in serum vials in the mechanical freezer at -35° C.

n. Tumor antigen-Lyl:--The last of these antigens was prepared from liver tumors which had been recovered from birds having naturally occurring visceral lymphomatosis. Filtrates, prepared from these tumors, had been used for the inoculation of chicks, producing tumor in a high percentage of cases indicating the presence of the agent (Burmester, B. R., oral communication). One hundred grams of the tumor was blended with 400 milliliters of 0.85 percent saline for five minutes, and the same procedure followed as was described for TA-L9.

B. Antibody Production

The antiserums used in the tests were produced in rabbits, guinea pigs, chickens and ducks by the inoculation of the various antigens. The route, volume and number of inoculations varied for the different antigens; however, the procedure used for the bleeding of each kind of animal remained constant. Also, the freezing and storage procedure was the same, regardless of the inoculum or animal involved.

a. Bleeding Procedure

Each animal was bled prior to inoculation to provide a control material by which the presence of non-specific reacting materials could be detected. One or more bleedings were made following the inoculations and the serums used as a source of antibodies. The serums obtained before the inoculations were classified as normal serums. By combining the symbols N for normal, R, GP, D, and C for rabbit, guinea pig, duck, and chicken, respectively, and S for serum, each of the serums could be easily identified; for example, NRS represented normal rabbit serum.

All bleedings were made aseptically by cardiac puncture. For the rabbits, a 30 milliliter syringe fitted with a 1-inch 18 gauge needle was used and approximately 30 milliliters of blood removed at each bleeding. With the guinea pigs, a 10 milliliter syringe and a $1\frac{1}{4}$-inch 20 gauge needle were used, while with the chickens and ducks, a 20 milliliter syringe fitted with a $1\frac{1}{4}$-inch 20 gauge needle was employed. Approximately 8 to 10 milliliters of blood was taken from the guinea pigs and 15 to 20 milliliters from the chickens and ducks. At the final bleedings, as much blood as possible was secured from each animal.

The bleed, after having been transferred from the syringe to the test tube, was allowed to clot at room temperature, and then thoroughly chilled by placing the tubes in a refrigerator at 4° C for two hours. They were then centrifuged at 1,000 g for 15 minutes to compress the clots and the serums transferred to serum tubes which were immediately sealed with a flame, and as soon as sufficiently cool, placed in an alcohol-dry ice bath at approximately -40° C. They were then placed in a mechanical freezer at -35° for storage. The procedure outlined here was used for both the pre- and post-inoculation bleedings.

b. Inoculation Procedure

Inoculation techniques varied for the different animals. The method used for each animal will be described and the volume and number of inoculations given later according to the individual antiserums.

1. Rabbits:--The inoculations were made intravenously into the marginal ear vein. The ears were washed thoroughly with soap and warm water and the area over the marginal vein shaved, rinsed with water and swabbed with cotton which had been dipped into 95 percent alcohol. A one milliliter tuberculin syringe and a 1-inch 24 gauge needle were used for the inoculations. After all the material had been injected, the needle was carefully removed, slight pressure applied to the area, the ear rinsed with cold water, dried with a soft cloth, and the rabbit returned to its cage.

2. Guinea pigs:--The intraperitoneal route was used for all of the inoculations in guinea pigs. The animal was placed on its back, with its legs extended, and the abdominal wall cleansed with 95 percent alcohol. The inoculations were made through the posterior portion of the abdominal wall, with the needle directed anteriorly. A one milliliter

uberculin syringe and a 1-inch 24 gauge needle were used for these noculations. No special care was needed in removing the needle or handing the animal before returning it to its cage.

3. Chickens and ducks:--The same procedure was used for the inoculation of both the chickens and ducks. The bird was placed on its side on a table and the area over the brachial vein picked clean of feathers. After cleansing the area with 95 percent alcohol, inoculation was made into the vein using a one milliliter tuberculin syringe and a 1-inch 24 gauge needle. Pressure was exerted on the area following the inoculation to control the hemorrhage.

c. Inoculations and Bleedings

The number and volume of injections will be described according to the antigen involved and animal being inoculated.

1. Influenza antigen:--Three rabbits were given two inoculations of one milliliter each at an interval of seven days. Fourteen days later, a third inoculation of 0.5 milliliters was given and bleedings started five days following this third inoculation. Three bleedings were made at seven-day intervals and the animals bled to death at the third bleeding.

Fifteen guinea pigs were given an initial inoculation of 0.5 milliliters and followed at five-day intervals by two inoculations of 0.25 milliliters each. Ten days after the last inoculation, bleedings were started and made every seven days with the surviving animals being bled to death at the fourth bleeding. Two of the animals died following the second bleeding and four following the third bleeding, as a result of excessive damage to the heart and hemorrhage into the pericardial sac.

Six chickens were given two inoculations of one milliliter each at n interval of seven days. Bleedings were started 10 days later and ontinued every seven days until three bleedings had been made. One of he birds died following the second bleeding and showed excessive cardiac amage. Two of the birds were bled to death at the third bleeding. By rror, three of the birds were bled to death at the first bleeding.

To facilitate the identification of these antiserums, the following ymbols were assigned:

FRS - rabbit anti-influenza serum

FGPS - guinea pig anti-influenza serum

FCS - chicken anti-influenza serum

The number of inoculations and bleedings and the amounts of serum btained are shown in Table VIa.

2. Normal tissue antigen:--Twenty four guinea pigs were given four noculations of 0.25 milliliters each at weekly intervals and 10 days llowing the last inoculation, they were bled. Three of the guinea igs died following the first bleeding. The 21 survivers were bled to eath seven days later.

Four chickens and two ducks were inoculated three times at weekly ntervals with 1.0 milliliter of the antigen. Ten days later, bleedings ere started and repeated every seven days, with all the birds being led to death at the third bleeding.

Symbols were assigned to these materials, as described previously, he only new item being the normal tissue antigen, which was designated T. These antiserums were thus designated as:

TABLE VIa

INOCULATIONS WITH INFLUENZA VIRUS

Animal	No.	Inoculation No.	Inoculation ml.	Inoculation Total ml.	Bleeding Before Inoc. No.	Bleeding Before Inoc. ml.*	Bleeding After Inoc. No.	Bleeding After Inoc. ml.*
Rabbit	35	2	1.0					
		1	0.5	2.5	1	15	3	173
	37	2	1.0					
		1	0.5	2.5	1	15	3	140
	50	2	1.0					
		1	0.5	2.5	1	15	3	175
Guinea Pig	15 pigs	1	0.5					
		2	0.25	1.0**	1	56	4	247
Chicken	K1321G	2	1.0	2.0	1	15	2	65
	K1316L	2	1.0	2.0	1	15	3	85
	K1319E	2	1.0	2.0	1	15	3	95
	$K1460C_4$	2	1.0	2.0	-	-	1	50
	$K1402G_2$	2	1.0	2.0	-	-	1	50
	$K717W_2$	2	1.0	2.0	-	-	1	50

* ml. of serum recovered
** total ml. for each guinea pig

TABLE VIb

INOCULATIONS WITH NORMAL TISSUE ANTIGEN

Animal	No.	Inoculation No.	Inoculation ml.	Inoculation Total ml.	Bleeding Before Inoc. No.	Bleeding Before Inoc. ml.*	Bleeding After Inoc. No.	Bleeding After Inoc. ml.*
Guinea Pig	24 pigs	4	0.25	1.0	1	126	2	300
Chicken	$K1310G_2$	3	1.0	3.0	2	40	3	70
	$K1310E_2$	3	1.0	3.0	2	42	3	85
	$K1310D_2$	3	1.0	3.0	2	37	3	70
	$K1413H_2$	3	1.0	3.0	2	40	3	75
Duck	5	3	1.0	3.0	1	10	3	163
	6	3	1.0	3.0	1	15	3	175

* ml. of serum recovered
** total ml. for each guinea pig

NTGPS - Guinea pig antinormal tissue serum

NTCS - Chicken antinormal tissue serum

NTDS - Duck antinormal tissue serum

tabulation of these inoculations and bleedings is shown in Table VIb.

3. Tumor antigens:--As was described previously, eight tumor antigens are prepared from liver tumor materials and one from pectoral tumor issue. The TA-L2 was used for the inoculation of guinea pigs, and the A-L1 for the inoculation of guinea pigs, ducks and chickens. All of he other tumor antigens were used for the inoculation of chickens, with he exception of the TA-L3 which was employed only as a test antigen. n Table VII the antigens and animals involved, the number and volume of noculations, the number of bleedings, and volume of serum recovered efore and after inoculation are tabulated.

The antiserums from these animals had to be identified, especially he chicken antiserums, and the following symbols were employed. T was sed to designate tumor antigen as the inoculated material, which was llowed by the symbol for the respective animal, such as GP for guinea ig, D for duck and C for chicken, and that was followed by S, indicating ntiserum. The symbol for the respective antigen as L1, L2, PTL, et tera, was then added. For example, the guinea pig antitumor-L1 serum as designated TGPS-L1.

The interval between inoculations and between the last inoculation nd the first bleeding varied. The guinea pigs were all inoculated at even-day intervals and the first bleeding made 10 days after the last noculation. The bleedings were then made at seven-day intervals with ll of the guinea pigs being bled to death at the second bleeding. This

TABLE VII

INOCULATIONS WITH TUMOR ANTIGENS

Inoculum	Animal	No.	Inoculation No.	Inoculation ml.	Inoculation Total ml.	Bleeding Before Inoc. No.	Bleeding Before Inoc. ml.	Bleeding After Inoc. No.	Bleeding After Inoc. ml.
TA-L1	Guinea Pig	24 pigs	4	0.25	1.0	1	178	2	305
	Chicken	K1412J_2	3	1.0	3.0	3	55	3	105
		K1401R_3	3	1.0	3.0	3	60	3	110
		K1301W	3	1.0	3.0	3	60	3	105
		K1310X	3	1.0	3.0	3	60	3	115
	Duck	1	3	1.0	3.0	1	10	3	150
		2	3	1.0	3.0	1	15	3	160
		3	3	1.0	3.0	1	13	3	165
		4	3	1.0	3.0	1	15	1	28
TA-L2	Guinea Pig	24 pigs	4	0.25	1.0	1	147	2	300
TA-PT4	Chicken	K1466C	9	2.0	18.0	1	25	2	36
		K1513I	9	2.0	18.0	1	20	2	40
		K2P95	9	2.0	18.0	1	20	2	40
TA-L4A	Chicken	K1503O	3	2.0	6.0	1	25	1	20
		K2P100	9	2.0	18.0	1	25	2	40
		K5P42	3	2.0	6.0	1	20	1	20
TA-L4B	Chicken	K2P93	9	2.0	18.0	1	20	2	40
		K5P46	9	2.0	18.0	1	20	2	40
TA-L4C	Chicken	K2P89	9	2.0	18.0	1	20	2	40
		K2P97	9	2.0	18.0	1	15	2	40
		K5P49	9	2.0	18.0	1	20	2	40
TA-L5A	Chicken	K2P99	11	2.0	22.0	1	22	2	50
		K2P96	11	2.0	22.0	1	25	2	50
		K1455Q	11	2.0	22.0	1	25	2	50
TA-L5B	Chicken	K5P52	11	2.0	22.0	1	25	2	50
		K1473Y	11	2.0	22.0	1	25	2	50
		K1473Z	11	2.0	22.0	1	25	2	50
TA-L5C	Chicken	K2P90	11	2.0	22.0	1	25	2	50
		K1414E_2	11	2.0	22.0	1	25	2	50
		K2P72	11	2.0	22.0	1	25	2	50
TA-L7A	Chicken	K2P99	2	2.0					
			1	4.0	8.0	-	-	1	50
		K1455Q_2	2	2.0					
			1	4.0	8.0	-	-	1	50
		K2P98	2	2.0					
			1	4.0	8.0	-	-	1	50
TA-L7B	Chicken	K2P42	2	2.0					
			1	4.0	8.0	-	-	1	17
		K1473Y	2	2.0					
			1	4.0	8.0	-	-	1	24
		K1414D_2	2	2.0					
			1	4.0	8.0	-	-	1	19

TABLE VII (Cont.)

Inoculum	Animal	No.	Inoculation No.	Inoculation ml.	Inoculation Total ml.	Bleeding Before Inoc. No.	Bleeding Before Inoc. ml.	Bleeding After Inoc. No.	Bleeding After Inoc. ml.
TA-L9	Chicken	$K1473L_2$	11	0.5					
			1	2.0	75	2	55	2	60
		$K14730_3$	11	0.5					
			1	2.0	7.5	2	55	2	60
		$K1516L_2$	11	0.5					
			1	2.0	7.5	1	30	2	60
		K620Q	10	0.5					
			1	2.0	7.0	1	25	3	50
		$K669L_2$	10	0.5					
			1	2.0	7.0	1	25	3	60
TA-Ly1	Chicken	K258K	11	0.5					
			1	2.0	7.5	2	70	2	60
		K262B	11	0.5					
			1	2.0	7.5	2	60	2	60
		$K669X_2$	11	0.5					
			1	2.0	7.5	1	45	1	60
		K1466M	11	0.5					
			1	2.0	7.5	2	55	1	60
		$K1511H_2$	11	0.5					
			1	2.0	7.5	1	35	2	60
		K350S	10	0.5					
			1	2.0	7.0	1	25	3	60
		K620B	10	0.5					
			1	2.0	7.0	1	25	3	60
		$K669C_3$	10	0.5					
			1	2.0	7.0	1	25	3	50

interval of seven days between bleedings was used routinely for all of the inoculated animals, the only variation being in the number of bleedings made.

The chickens and ducks receiving the TA-L1 were inoculated at weekly intervals and the bleedings started seven days after the last inoculation. The chickens receiving the TA-PTL, TA-LLA, B, and C, and TA-L5A, B, and C, however, were inoculated twice weekly and the first bleedings made 10 days after the last inoculation. Two of the birds receiving the TA-LLA received only three inoculations instead of nine, as was the case with the other birds in this group. The chickens receiving TA-L9, and TA-Ly 1 were inoculated twice weekly with 0.5 milliliters for a total of 11 inoculations, and then given the final inoculation of 2.0 milliliters after an interval of 10 days. The TA-L7A and B received two inoculations of 2.0 milliliters and a third inoculation of L.0 milliliters at intervals of seven days.

L. Infected chicken serum:--The serums of infected birds would not contain the agent in as concentrated amounts as the centrifuged materials. Therefore, a larger number of inoculations was needed to supply a comparable amount of agent for antibody stimulation. Six guinea pigs and two ducks were inoculated 15 and 14 times, respectively, at weekly intervals. The first two inoculations contained four milliliters each; the volume was then reduced to one milliliter for the remaining inoculations. Seven days after the last inoculations, they were bled. The ducks were bled to death at the first bleeding, while the guinea pigs were bled to death at the second bleeding. These antiserums were designated:

ICSGPS - Guinea pig antinormal chicken serum serum

ICSDS - Duck antinormal chicken serum serum

The tabulations of the inoculations and bleedings with the infected and normal chicken serums are shown in Table VIII.

TABLE VIII

INOCULATIONS WITH CHICKEN SERUMS

Inoculum	Animal	No.	Inoculation			Bleeding			
			No.	ml.	Total ml.	Before Inoc.		After Inoc.	
						No.	ml.*	No.	ml.*
Infected Chicken Serum (ICS)	Guinea Pig	6 pigs	2	4.0					
			13	1.0	21.0**	1	48	2	105
	Duck	74	2	4.0					
			12	1.0	20.0	1	30	1	80
		75	2	4.0					
			12	1.0	20.0	1	30	1	80
Normal Chicken Serum (NCS)	Guinea Pig	6 pigs	2	4.0					
			13	1.0	21.0**	1	45	2	100
	Duck	71	2	4.0					
			12	1.0	20.0	1	30	1	80
		73	2	4.0					
			12	1.0	20.0	1	30	1	80

* ml. of serum recovered

** total ml. for each guinea pig

VI. STANDARDIZATION OF TECHNIQUE

A reaction involving a known virus and specific antiserum was needed for the standardization of the technique with regard to the order of addition of the reagents and the temperature and duration of the incubation periods. The influenza virus was chosen to serve as the antigen, and antiserums were produced in rabbits and chickens. The rabbit anti-influenza serum was titrated first and then the antigen standardized at a concentration where it was the limiting factor with regard to the amount of complement fixed. This was necessary to insure that all of the activity of the chicken anti-influenza serum could be measured when it was allowed to react with the antigen in an indirect test.

The direct reaction involving the influenza antigen and rabbit anti-influenza serum was standardized and an indirect test on the chicken anti-influenza serum attempted. If influenza antibodies were present in the chicken anti-influenza serum, they would combine with the antigen and reduce the amount of complement fixed when the rabbit anti-influenza serum was added. However, a larger amount of complement was fixed when the chicken anti-influenza serum was present than occurred in the direct reaction. Normal chicken serum, from the same birds furnishing the anti-influenza serum, was then tested and similar results obtained, with even greater amounts of complement fixed than was obtained with the chicken anti-influenza serum. Normal serums from horses, cows and ducks were tested but failed to affect the amount of complement fixed by the direct reaction of influenza virus and rabbit anti-influenza serum. Control tests in which rabbit anti-influenza serum was tested against chicken

anti-influenza serum and normal chicken serum showed there was a reaction occurring in both instances which produced a complex capable of fixing approximately 7.5 units of complement. This fixation was considered due to an antigenic material in the chicken serums and specific antibodies in the rabbit anti-influenza serum because, as has been mentioned, antibodies produced in the chicken are not capable of producing a complement-fixing complex with specific antigen.

When the indirect titers of the chicken anti-influenza serum and normal chicken serum were calculated, a negative rather than positive value was obtained. The magnitude of this negative titer increased with increased dilution of the chicken anti-influenza serum but decreased with the higher dilution of the normal chicken serum. This difference was attributed to the following facts. Three reactions occurred in the tests where the chicken anti-influenza serum was used. The first was the reaction of the chicken anti-influenza serum with the influenza antigen, which would reduce the amount of antigen available to react with specific antibodies in the rabbit anti-influenza serum and decreased the final amount of fixation. The second reaction was between the rabbit anti-influenza serum and the antigen which caused fixation in proportion to the amount of antigen available. The third reaction was the one between the unknown component in the chicken anti-influenza serum and an antibody in the rabbit anti-influenza serum. This third reaction was apparently capable of fixing complement and would add to the final amount of fixation measured. When normal chicken serum was present, only two of the reactions occurred; the one between the rabbit anti-influenza serum and the influenza antigen, and the one between the unidentified

antibodies in the rabbit anti-influenza serum and the specific component present in the chicken serums. Both of these reactions fixed complement. The third reaction, present only when the chicken anti-influenza serum was used, acted in the opposite way in that it decreased the amount of fixation by its action on the influenza antigen. As the chicken anti-influenza dilution was increased, this reaction was lessened and the effect of the other two reactions became more evident.

A determination of the nature of the substance, present as a normal constituent of the chicken serums, was attempted. The antibodies in the rabbit anti-influenza serum which reacted with this component apparently were produced by a substance in the influenza antigen other than the influenza virus. The influenza antigen had been grown in chicken embryos, and harvested from the allantoic fluid of these embryos at approximately their fourteenth day of incubation. When concentrated by centrifugation, a portion of the allantoic fluid would necessarily accompany the virus particles, and apparently contained the material responsible for the production of the antibodies which reacted with the constituent in the chicken serums.

The possibility that this was a heterophile-type reaction was considered. The heterophile antigens are capable of stimulating antibody production when inoculated into rabbits (Zinsser, et al., 1945). It has been shown by Schechtman (1948) that early embryonic stages of the chicken contain one or more antigens which occur in the adult chicken serum. Krichevsky (1923), using aqueous suspensions, detected heterophile antigen in the embryo as early as the fourth day of incubation. This same antigen is known to be present in the organs and blood of

chickens, which would account for the type of reaction encountered when antiserum, produced by the inoculation of embryo material, was allowed to react with normal or immune chicken serums. The rabbit is one of the few animals capable of producing these heterophile antibodies (Zinsser et al., 1945). Animals which have the heterophile antigen in either their organs or blood do not produce antibodies when inoculated with the heterophile antigen. If the assumption were correct that the interfering substance in the indirect tests was due to a heterophile antigen-antibody reaction, the use of guinea pigs for the production of complement-fixing antiserum for the direct reaction should eliminate this difficulty, because guinea pigs have this antigen in their organs.

Guinea pig anti-influenza serum was tested against chicken anti-influenza serum and normal chicken serum to determine if a reaction occurred when they were combined, as had been the case with the rabbit anti-influenza serum. No evidence of such a reaction was noted, indicating that the guinea pigs had not formed antibodies which would react with the component in the chicken serums, and thus should not give negative titers for indirect tests on chicken anti-influenza serum. The indirect titer for the chicken anti-influenza serum, when tested with the rabbit anti-influenza serum, gave negative values ranging from -0.2 units to -2.7 units. However, with the use of guinea pig anti-influenza serum in the direct reaction, positive values up to 11.5 units were obtained. The chicken anti-influenza serum and rabbit anti-influenza serum had been shown to fix 7.5 units of complement. This fixation would occur at the same time that the influenza antigen and specific antibodies in the rabbit antiserum were combining and fixing complement. The

fixation measured would thus be the total of these two reactions, and if the fixation brought about by the reaction of the chicken and rabbit anti-influenza serums were subtracted from the total amount of complement fixed in the indirect reaction, the resultant value would be the amount of complement fixed by the influenza antigen and specific antibodies from the rabbit anti-influenza serum. If the 7.5 units were subtracted from the units of complement fixed in the indirect reaction and the resultant value subtracted from the direct reaction value, a correct indirect titer for the chicken antiserum should be obtained. When this was done, chicken anti-influenza serum titers up to 7.3 units were obtained, which agreed closely with the titers found when the guinea pig anti-influenza serum was used. This further indicated that the interfering reaction believed to be of a heterophile type was a distinct entity and independent of any of the other reactions taking place. A tabulation of the preceding reactions and calculations is given in Table IX.

From these findings it was considered necessary to use guinea pigs for the production of the antiserums for any reactions involving chicken tissue components, since all of the antigens for the various tests had been prepared from chicken tissues, either normal or tumorous, and would contain the heterophile antigen.

The temperature and length of incubation recommended by Rice (1946) had been used in the tests for the detection of the heterophile antigen-antibody reaction. The influenza antigen, guinea pig anti-influenza serum and chicken anti-influenza serum were used to test the technique procedure.

TABLE IX

INDIRECT TESTS ON CHICKEN ANTI-INFLUENZA SERUM WITH RABBIT AND GUINEA PIG ANTI-INFLUENZA SERUMS IN THE DIRECT REACTIONS

Reaction	Antigen Dilution	Units Fixed	Chicken Serum Titer
FA + FRS*	1:20	14.4	-
	1:40	9.4	-
	1:80	6.8	-
FA + FGPS*	1:20	25.2	-
	1:40	16.1	-
	1:80	9.5	-
FA + FRS + FCS*	1:20	14.6	-0.2
	1;40	11.2	-1.8
	1:80	9.5	-2.7
FA + FGPS + FCS	1:20	17.8	7.4
	1:40	10.2	5.9
	1:80	5.3	4.2
FRS + FCS	-	7.5	-
FRS + NCS	-	7.1	-
FGPS + FCS	-	0.0	-
FGPS + NCS	-	0.0	-
(FA + FRS + FCS) - (FRS + FCS)	1:20	7.1	7.3
	1:40	3.7	5.7
	1:80	2.0	4.8

* at a dilution of 1:5

The order in which the various reagents should be added, with reference to the incubation periods, was checked first. For the indirect test, three incubations were needed; the first allowed the chicken antiserum to react with the antigen; the second permitted the guinea pig antiserum and antigen to combine and act on the complement; and the third allowed complement to act on the sensitized cells. In the following discussion these will be referred to as the first, second, and third incubations.

The advisability of adding the complement before the first incubation, or adding it at the same time as the guinea pig antiserum, was checked. Either method would allow the antigen-guinea pig antiserum complex to act upon the complement; however, a portion of the complement might be destroyed during the first incubation, if it were added before this time. Another method, in which all the reagents were mixed at the same time, was investigated. This would completely eliminate the first incubation period. A fourth method consisted of mixing the antigen, guinea pig antiserum, and complement before the first incubation and then adding the chicken antiserum. This would determine if the chicken antiserum could cause the breaking of the union between the antigen, guinea pig antiserum, and complement, and thus combine with the antigen.

Method number 1, where complement was added before the first incubation, gave an indirect titer for the chicken antiserum of 9.1 units. In the second method, when the complement was not added until after this incubation, the chicken antiserum titer was 9.8 units. This would indicate that the antigen and chicken antiserum combined equally as well either in the presence or absence of complement. The addition of the

complement after the first incubation was more desirable, since tests on the temperature and time of incubation for the antigen-chicken antiserum reaction were to be made and the addition of the complement after their incubation would eliminate the possibility of destruction of a portion of it due to varying times and temperatures employed. When all the reagents were added at one time as in the third method, the chicken antiserum titer was only 6.1. This reduction in the titer was considered due to the guinea pig antiserum combining with the antigen at the same time as the chicken antiserum, thus preventing the chicken antiserum from exerting all of its activity. In the fourth method, where the chicken antiserum was added after the antigen, guinea pig antiserum, and complement had been incubated, no effect by the chicken antiserum was detected. Therefore, it was concluded that the antigen-guinea pig antiserum-complement complex was stable and could not be reversed by the presence of the chicken antiserum. From these results, method 2 was chosen to be used in subsequent tests.

The effect of the time and temperature of incubation on the indirect titer of the chicken antiserum was then determined. The incubation of the antigen-chicken antiserum mixture was carried out at 37° C for periods of 15 minutes, 30 minutes, one hour, and two hours, and at 4° C for periods of one, two, four and 16 hours. Thirty minutes at 37° C was selected to be used for the first incubation period in the standard technique. The second incubation, after the addition of the guinea pig antiserum, was then tested, using various times at temperatures of 4° and 37° C. Two hours at 37° C gave the optimum results. Thirty minutes at 37° C was found best for the third incubation, after the addition of the sensitized red blood cells. The standard technique proceeded as follows

Antigen + chicken antiserum

30 minutes - 37° C

Guinea pig antiserum + complement

2 hours - 37° C

Sensitized red blood cells

30 minutes - 37° C

For direct tests of a specific reaction the first incubation was not necessary. Therefore, the antigen, antiserum, and complement were all added at the same time, incubated two hours at 37° C, the sensitized red blood cells added and then the final 30-minute incubation before the electrophotometer readings were made. If the direct test was in conjunction with an indirect test, however, the antigen was incubated 30 minutes at 37° C, or the first incubation, the same as the antigen-chicken antiserum mixture of the indirect test. This made the only difference the presence of the chicken antiserum in the indirect reaction.

The volume of complement and ionic buffer varied according to the amount of complement desired in the individual tube. The antigen and antiserums were always used in volumes of 0.1 milliliter. In direct tests, the complement and buffer occupied 0.8 milliliter and in the indirect tests where chicken serum was being tested, they occupied 0.7 milliliter. One milliliter of sensitized cells was used, making the total volume 2.0 milliliters.

VII. RESULTS AND DISCUSSION

The demonstration of a reaction between an antigen and an antibody by the complement fixation test depends upon the inactivation of a portion of the complement following the formation of an antigen-antibody complex. If one of these reagents, either antigen or antibody, is of known composition, and has been shown to be active when employed in a complement fixation test, it can be used for the detection of the other reagent. This means that if an antigen of known quality is used, specific antibodies can be demonstrated, or if a known antibody is available, the presence of specific antigen can be determined.

When a preparation contains a particular substance which is desired for use as an antigen in the complement fixation test, but no method is available for the quantitative measurement of the substance, its inoculation into guinea pigs may stimulate antibody formation. If this antiserum is allowed to react with the antigenic material, a demonstrable reaction may occur. A situation of this type exists in the case of the agent of visceral lymphomatosis. The only method by which the presence of the agent can be demonstrated is the inoculation of chickens at one day of age. These birds must be maintained for 200 to 300 days and the lymphoid tumors, developing as a result of the inoculation, used as a measure of the amount of agent that was present in the original inoculum. The presence of natural infection in the inoculated chickens, the space required to house sufficiently large groups of birds, and the 200-300 day period necessary for tumor development, limit the amount of work which can be directed along this line. Therefore, the development of a technique by

which the presence of the agent of visceral lymphomatosis could be detected, and with which its potency or reactivity could be measured, would be of great value in studies on this disease. The tests reported here were conducted in an attempt to develop such a technique.

a. Tests for the RPL strain 12 agent or specific antibodies in infected chicken serum:--The serums of chickens possessing visceral lymphoid tumors were known to contain the agent of visceral lymphomatosis (Burmester, 1947b). The RPL Strain 12 visceral tumor had been transmitted by the inoculation of the serums of infected birds (Burmester et al., 1946), indicating the agent was present in the serums of such birds and the inoculation of these serums into guinea pigs should, theoretically, stimulate the formation of antibodies which would react with the agent. If guinea pig antiserums were produced by the inoculation of the serums of infected birds and mixed with chicken serum which contained the agent, an antigen-antibody complex might be formed that would be detectable by the complement fixation test. To determine if such a reaction would occur, the following tests were conducted.

Guinea pig antiserums were produced by the inoculation of normal and infected chicken serums. The guinea pig antinormal-chicken serum serum was prepared to serve as a control on any reaction which might occur between normal tissue components (other than the heterophile materials) of the infected chicken serum and antibodies that had been stimulated by them. The only known difference between the normal and infected chicken serums was the presence of the agent in the latter. If the normal tissue constituents of the infected chicken serum stimulated antibody formation, a normal tissue reaction would occur. By using normal chicken serum

antigen and guinea pig antinormal-chicken serum serum as control reagents, the amount of fixation which should be attributed to these materials could be determined.

A preliminary test was then made to determine if any fixation of complement would result from the reaction of different combinations of these reagents. This preliminary test was not designed to measure the exact amount of fixation taking place, but would indicate when significant amounts of fixation had occurred. If fixation had taken place, the reagents involved could then be titrated against varying amounts of complement and the exact magnitude of the fixation determined. The use of a preliminary test provided not only a relatively simple method for the demonstration of fixation but reduced greatly the amounts of the antigens and antiserums required which, in some instances, were quite limited.

The procedure for this preliminary test consisted of placing together 0.1 milliliter of each of the test reagents (antigen and antiserum), 0.2 milliliter of complement and 0.6 milliliter of ionic buffer. These were incubated two hours at 37° C, during which time the tubes were plugged with cork steppers. This was done routinely to prevent any reduction in volume due to evaporation. Following this incubation, 1.0 milliliter of sensitized red blood cells was added to each tube, the steppers replaced, tubes shaken thoroughly and again incubated for 30 minutes at 37° C. They were then removed from the water bath and the electrophotometer readings determined.

By knowing the anticomplementary activity of each of the reagents, the amount of hemolysis which should occur in a given tube, providing there was no fixation of complement, could be estimated. Three units of

complement was used in each tube. Complement titrations gave a value of 0.0025 milliliter of undiluted complement, or 0.25 milliliter of a 1:100 dilution to be necessary for one unit. Three units, when using a 1:100 dilution, would have required 0.75 milliliters, but this volume was too large for rapid pipetting. It was calculated that 0.2 milliliter of a 1:15 dilution would contain 0.0075 milliliter of undiluted complement, which was the equivalent of three units. The accuracy of this calculation was checked by diluting 0.2 milliliter of a 1:15 dilution with 0.4 milliliter of ionic buffer. Each 0.2 milliliter of this material should contain one unit, or sufficient complement to produce exactly 50 percent hemolysis. When added to 0.8 milliliter of buffer and 1.0 milliliter of sensitized red blood cells, from 48 to 52 percent hemolysis was produced, indicating the calculation to be correct.

The guinea pig antiserums produced by the inoculation of the normal and infected chicken serums were then tested using the normal chicken serum (NCSA) and infected chicken serum (ICS) as antigens. Two-fold dilutions, ranging from 1:2 to 1:16 were prepared for each of the reagents. All of the dilutions of each antiserum were tested against each dilution of both antigens and gave the results shown in Table X. If there had been no fixation or anticomplementary activity in any of the reactions, all of the readings would have been 70. In some of the higher concentrations, however, the anticomplementary activity of the reagents had inactivated a portion of the complement, reducing the amount available to hemolyze the red cells and thus lower the readings. In the tube containing both the NCSA and NCSGPS at dilutions of 1:2, only slightly over one unit of complement remained active, as indicated by the reading of

TABLE X

PRELIMINARY TESTS WITH POOLED GUINEA PIG ANTINORMAL AND ANTI-INFECTED CHICKEN SERUM SERUMS AGAINST POOLED NORMAL AND INFECTED CHICKEN SERUM ANTIGENS

Antigen	Dilution	Antiserum NCSGPS 1:2	NCSGPS 1:4	NCSGPS 1:8	NCSGPS 1:16	ICSGPS 1:2	ICSGPS 1:4	ICSGPS 1:8	ICSGPS 1:16
		Electrophotometer Readings							
NCSA	1:2	36	63	70	70	41	66	70	70
	1:4	61	70	70	70	68	70	70	70
	1:8	70	70	70	70	70	70	70	70
	1:16	70	70	70	70	70	70	70	70
ICS	1:2	43	68	70	70	39	62	70	70
	1:4	65	70	70	70	66	70	70	70
	1:8	70	70	70	70	70	70	70	70
	1:16	70	70	70	70	70	70	70	70

36. The anticomplementary value for the 1:2 dilution of the NCSA was 0.9 units and for the NCSGPS it was 0.7 units. This totaled 1.6 units, which was the amount of complement non-specifically destroyed, leaving only 1.4 of the original three units available to be acted upon by a specific antigen-antibody reaction. Since there was slightly over one unit of complement active against the red cells, as indicated by the reading of 36, no specific reaction could have taken place. In the higher dilutions where the anticomplementary activity was less, the amount of complement remaining active, as measured by the degree of hemolysis, increased proportionally. All combinations of the various reagents followed this pattern. If a specific reaction of any magnitude had taken place, all or a large part of the complement, not accounted for by the anticomplementary activity of the reagents, would have been rendered inactive and the amount of hemolysis would have been markedly reduced or completely absent. It was evident, therefore, that either there was no antibody formation in the guinea pigs, or the normal and infected chicken serums did not function as satisfactory antigens.

The guinea pig antiserums had been pooled samples in the tests just described. There remained the possibility that one or more of these antiserums, when tested separately, might exhibit the desired reaction. If antibodies had been formed by one or two of the guinea pigs, the dilution resulting from the pooling might have made their detection impossible. Therefore, each guinea pig antiserum was tested in the same manner as described for the pooled samples, with the exception that only the 1:2 and 1:4 dilutions were used. None of the individual antiserums, however, gave evidence of a specific reaction having taken place, all of

the reduction in fixation being accounted for as anticomplementary activity. The results obtained when the individual antiserums were tested against the NCSA and ICS are shown in Table XI.

It was concluded that either the agent particles in the infected chicken serum were not present in sufficient concentration to stimulate antibody formation, when inoculated into guinea pigs, or if antibodies had been formed, the concentration of the particles in the ICS was not sufficient to allow a detectable reaction to take place when it was used as an antigen. If the former were true, and no antibodies had been formed, no reaction could be expected. However, if the agent had stimulated antibody formation but was not capable of forming a detectable antigen-antibody complex when in contact with these antibodies, the substitution of a concentrated suspension of the agent as an antigen should result in a measurable reaction.

The tumor antigen-L1 was found to be highly anticomplementary, destroying over three units of complement in dilutions as high as 1:8. The results which might be obtained in tests where a material having such a high anticomplementary activity was used, could not be depended upon, and another material was selected as a source of agent. The TA-L2 (tumor antigen - liver 2) which had been prepared from the same materials, but purified to a higher degree, was tested and found to have only slight anticomplementary activity, the maximum being 0.5 units at a dilution of 1:2. This antigen (TA-L2), having been prepared from tumorous liver material, contained some normal chicken tissue components. These components might react with normal tissue antibodies that had been formed in the guinea pigs following the inoculations with the chicken serums, and

TABLE XI

PRELIMINARY TEST OF POOLED NORMAL AND INFECTED CHICKEN SERUM ANTIGENS AGAINST INDIVIDUAL GUINEA PIG ANTINORMAL AND ANTI-INFECTED CHICKEN SERUM SERUMS

Antiserum	Guinea Pig No.	Dilution	Antigen NCSA 1:2	Antigen NCSA 1:4	Antigen ICS 1:2	Antigen ICS 1:4
			Electrophotometer Readings			
NCSGPS	1	1:2	39	58	38	57
		1:4	48	70	51	70
	2	1:2	36	61	40	62
		1:4	46	70	56	70
	3	1:2	42	59	43	60
		1:4	61	70	50	70
	4	1:2	32	50	35	56
		1:4	44	70	43	70
	5	1:2	52	70	49	68
		1:4	70	70	56	70
	6	1:2	30	51	36	59
		1:4	43	70	48	70
ICSGPS	7	1:2	41	61	42	63
		1:4	56	70	61	70
	8	1:2	45	63	40	64
		1:4	58	70	56	70
	9	1:2	39	57	39	54
		1:4	56	70	54	70
	10	1:2	43	64	42	62
		1:4	63	70	57	70
	11	1:2	40	67	46	64
		1:4	61	70	60	70
	12	1:2	44	61	46	58
		1:4	59	70	54	70

the NTA (normal tissue antigen) was used to serve as a control for such a reaction.

By testing the guinea pig antinormal chicken-serum serum and guinea pig anti-infected chicken-serum serum against both the NTA and the TA-L2, any specific agent reaction which might be present could be detected. The reaction between the NTA and NCSGPS determined the normal tissue antibody content of the guinea pig antinormal chicken-serum serum. By then testing the TA-L2 against this same antiserum, the normal tissue content of the TA-L2, in proportion to the amount found in the NTA, could be determined. The reaction between the ICSGPS and the NTA indicated the normal tissue antibody content of the guinea pig anti-infected chicken-serum serum compared to that of the guinea pig antinormal chicken-serum serum. From these values, the amount of normal tissue reaction which should occur when the ICSGPS was tested against the TA-L2 could be estimated, and when subtracted from the amount of reaction actually taking place, would indicate if any specific agent-agent antibody reaction had occurred. The anticomplementary activity for all of these reagents was determined. The largest amount of complement which would be non-specifically destroyed by the combination of any of these reagents was 1.2 units when the 1:2 dilution NTA was in contact with the same dilution of NCSGPS. Therefore, when the preliminary type of test was used, employing three units of complement, all tubes would have at least 1.8 units of complement remaining active and would give readings near 70 if no fixation by a specific reaction took place. As shown in Table XII, some fixation apparently had taken place in the reactions involving the lower dilutions of all of the reagents. To determine the extent of these

TABLE XII

PRELIMINARY TEST OF GUINEA PIG ANTINORMAL AND ANTI-INFECTED CHICKEN SERUM SERUMS AGAINST TA-L2 AND NT ANTIGENS

Antigen	Dilution	Antiserum							
		NCSGPS				ICSGPS			
		1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16
		Electrophotometer Readings							
NTA	1:2	14	46	70	70	9	43	66	70
	1:4	39	63	70	70	44	64	70	70
	1:8	70	70	70	70	70	70	70	70
	1:16	70	70	70	70	70	70	70	70
TA-L2	1:2	27	61	70	70	34	64	70	70
	1:4	56	70	70	70	60	70	70	70
	1:8	70	70	70	70	70	70	70	70
	1:16	70	70	70	70	70	70	70	70

reactions, the 1:2 and 1:4 dilutions were titrated in a direct reaction employing varying amounts of complement. Three sets of titrations were made, and the average number of units fixed is shown in Table XIII. The NTA + NCSGPS reaction fixed 1.4 units when both reagents were used at a 1:2 dilution, indicating that normal tissue antibodies, although in a low concentration, had been produced following the inoculation of the NCSA. The ICSGPS fixed 1.9 units when tested against the NTA, or 0.5 units more than the NCSGPS, showing that the concentration of normal tissue antibody was slightly greater in the ICSGPS than in the NCSGPS. However, when the antiserums were tested against the tumor antigen, the ICSGPS produced slightly less fixation than the NCSGPS, indicating that no specific agent-agent antibody reaction had taken place.

The tests just described had been conducted using pooled samples of the guinea pig anti-infected and antinormal chicken-serum serums. The guinea pig antiserums which made up these pooled samples were then tested against the tumor antigen-L2 and normal tissue antigen. The results obtained with these individual antiserums are shown in Table XIV and were practically the same as these obtained with the pooled samples, with the ICSGPS + TA-L2 reactions consistently showing the least fixation. This eliminated the possibility of utilizing the guinea pig antiserums produced by the inoculation of the infected chicken serum as a source of visceral lymphomatosis agent antibody.

The failure of the infected chicken serum to stimulate antibody formation did not eliminate the possibility that the agent, known to be present in this infected serum, might be detected by complement fixation. The presence of specific agent antibodies in the guinea pig

TABLE XIII

TITRATION OF GUINEA PIG ANTINORMAL AND ANTI-INFECTED CHICKEN SERUM SERUMS AGAINST TA-L2 AND NT ANTIGENS

Antigen	Antigen dil.	Test No.	Antiserum NCSGPS 1:2	NCSGPS 1:4	ICSGPS 1:2	ICSGPS 1:4
			Units Fixed			
NTA	1:2	(1)	1.6	1.0	1.8	0.9
		(2)	1.3	1.0	1.9	0.8
		(3)	1.2	0.7	1.9	1.2
		Ave.	1.4	0.9	1.9	1.0
	1:4	(1)	0.8	0.3	0.7	0.3
		(2)	1.0	0.3	0.9	0.2
		(3)	0.9	0.5	0.7	0.3
		Ave.	0.9	0.4	0.8	0.3
TA-L2	1:2	(1)	1.3	0.2	0.9	0.0
		(2)	1.0	0.2	0.7	0.1
		(3)	1.1	0.3	0.8	0.1
		Ave.	1.1	0.2	0.8	0.1
	1:4	(1)	0.3	0.1	0.1	0.0
		(2)	0.4	0.0	0.3	0.1
		(3)	0.4	0.1	0.2	0.0
		Ave.	0.4	0.1	0.2	0.0

TABLE XIV

TITRATION OF INDIVIDUAL GUINEA PIG ANTINORMAL AND ANTI-INFECTED CHICKEN SERUM SERUMS AGAINST TA-L2 AND NT ANTIGENS

Antiserum	Guinea Pig No.	Dil.	Antigen NTA 1:2	NTA 1:4	TA-L2 1:2	TA-L2 1:4
NCSGPS	1	1:2	1.6 *	0.8	1.0	0.2
		1:4	1.1	0.5	0.4	0.0
	2	1:2	1.7	1.3	1.1	0.4
		1:4	1.0	0.6	0.6	0.2
	3	1:2	0.9	0.2	0.8	0.3
		1:4	0.3	0.0	0.5	0.1
	4	1:2	1.2	0.6	1.1	0.5
		1:4	0.5	0.2	0.4	0.0
	5	1:2	1.8	1.3	1.4	0.9
		1:4	1.1	0.7	0.6	0.3
	6	1:2	1.1	0.6	0.7	0.4
		1:4	0.6	0.2	0.5	0.1
ICSGPS	7	1:2	1.7	1.2	1.1	0.2
		1:4	0.7	0.4	0.3	0.0
	8	1:2	2.1	1.3	1.4	0.7
		1:4	1.4	0.7	0.8	0.2
	9	1:2	1.1	0.4	0.8	0.1
		1:4	0.5	0.1	0.2	0.0
	10	1:2	1.4	0.7	1.0	0.3
		1:4	0.6	0.2	0.2	0.0
	11	1:2	0.6	0.1	0.2	0.0
		1:4	0.2	0.0	0.0	0.0
	12	1:2	1.7	1.1	0.9	0.3
		1:4	1.2	0.6	0.5	0.1

* Units fixed

anti-infected chicken-serum serum was dependent upon the presence of significant amounts of agent in the infected chicken serum used as the inoculum. As has been shown, no antibodies could be detected; however, if an antiserum were produced which was high in specific tumor agent antibody content, and it was allowed to react with the infected chicken serum, the presence of the agent might be detectable. In other words, the concentration of the agent in the ICS (infected chicken serum), although not in sufficient concentration to stimulate antibody formation, might, when in contact with a potent specific antibody, combine with it and fix complement. This possibility, although not considered to be likely, was of sufficient importance to warrant investigation.

Antiserums were produced in guinea pigs by the inoculation of concentrated suspensions of the agent. The TA-L1 (tumor antigen - liver 1) and TA-L2 (tumor antigen - liver 2) were used for this purpose. A third group of guinea pigs was inoculated with NTA (normal tissue antigen) to serve as a control on the normal tissue reactions of the tumor materials. The guinea pig antitumor serums were designated as TGPS-L1 and TGPS-L2. These two guinea pig antitumor serums, and the guinea pig antinormal tissue serum were used in tests employing normal and infected chicken serums as antigens. If the agent in the infected chicken serum combined with specific antibody, the tests employing the guinea pig antitumor serums and infected chicken serum would fix larger amounts of complement than the control reactions involving the normal tissue reagents.

As had been the procedure previously, a preliminary test was made using three units of complement and pooled antiserums. The results of these preliminary tests failed to indicate the presence of a reaction

involving the agent. However, some normal tissue reaction apparently took place in these tests where the NTGPS and TGPS-L1 (antiserums) and NCSA (antigen) were present. The TGPS-L2 exhibited little or no normal tissue reaction. This was attributed to the relative purity of the antigen (TA-L2) used in its production.

Titrations, using varying amounts of complement, to determine the exact amount of complement fixed were then conducted. The results of these tests, shown in Table XV, further indicated that no agent reaction had occurred and that the fixation which had taken place was due to normal tissue components in the antigen and specific antibodies in the various antiserums.

From the results of all the tests thus far described, it was concluded that, with the techniques employed, the presence of the agent in infected chicken serum could not be detected.

There remained the possibility that the infected chicken serum might contain antibodies which would react with a concentrated suspension of the agent, such as the tumor antigen-L2. Burmester (1947c) had shown that the serums of birds hyperimmunized against lymphoid tumor cells markedly reduced the mortality in birds inoculated with a suspension of viable tumor cells of the same type. The serums of chickens that had received injections of suspensions of apparently normal lymphoid tissue did not retard the growth of the tumor cells. Olsen (1947), using various tissues from birds possessing lymphoid tumors, obtained similar results. Therefore, the chicken is capable of producing a protective mechanism, presumably an antibody, against the lymphoid tumor cells. The production of such a protection against the agent of visceral lymphomatosis,

TABLE XV

TITRATION OF NORMAL AND INFECTED CHICKEN SERUM ANTIGENS WITH POOLED GUINEA PIG ANTINORMAL TISSUE, ANTITUMOR-L1 AND ANTITUMOR-L2 SERUMS

Antiserum	Dil.	Antigen NCSA 1:2	NCSA 1:4	NCSA 1:8	ICS 1:2	ICS 1:4	ICS 1:8
				Units Fixed			
NTGPS	1:2	2.1	1.2	0.4	2.0	0.8	0.2
	1:4	1.3	0.3	0.0	1.1	0.4	0.0
	1:8	0.3	0.0	0.0	0.3	0.0	0.0
TGPS-L1	1:2	1.6	0.9	0.3	1.7	0.8	0.3
	1:4	0.6	0.3	0.1	0.4	0.1	0.0
	1:8	0.2	0.1	0.0	0.0	0.0	0.0
TGPS-L2	1:2	0.5	0.1	0.0	0.4	0.3	0.0
	1:4	0.3	0.0	0.0	0.1	0.0	0.0
	1:8	0.0	0.0	0.0	0.0	0.0	0.0

and especially the RPL Strain 12 tumor, has not been reported. Rous (1913) in observations with a fowl sarcoma, described a natural resistance in some chickens to the inoculation of the tumor producing agent. Duran-Reynals (1940), working with the same agent and other agents producing similar types of tumors, found the natural resistance increased with the age of the chicken, but he could not determine the specificity of the neutralizing material. Friedwald and Kidd (1945) demonstrated, by complement fixation, antibodies in the blood of rabbits carrying various transplanted tumors. They attributed the reactions to what they termed "induced tissue antibodies" in that the reaction involves constituents of the various cells and not a causative agent. MacKenzie and Kidd (1945) differentiated between these induced tissue antibodies and an antibody that reacts specifically with a distinctive sedimentable constituent of Brown-Pearce carcinoma cells, but this constituent was not associated with the specific agent.

b. Standardization of a direct complement fixation test for use in testing for tumor agent antibodies in chicken serums:--The infected chicken serum (ICS) had been used previously as an antigen, but was now to be employed as an antiserum to determine if the agent, when stimulating the formation of visceral tumors, also stimulates antibody formation. If such an antibody were present, the indirect reaction would have to be used for its detection. A positive direct reaction, involving an antigen high in visceral tumor agent content, and a specific guinea pig antitumor agent serum would be necessary. Such a direct reaction could then be used in testing chicken serums for their agent antibody content by the indirect complement fixation technique previously described.

Antiserums to be used in the tumor direct test were prepared by inoculating guinea pigs with tumor antigens (TA-L1 and TA-L2) and the normal tissue antigen (NTA). The guinea pig antitumor antigen serums (TAGPS-L1 and TAGPS-L2) were to serve as sources of specific agent antibody, but normal tissue antibody formation was expected and the guinea pig antinormal tissue serum (NTGPS) was prepared to serve as a control. The three antigens, TA-L1, TA-L2, and NTA, used for the production of antibodies, also were used as antigens for the direct test standardization.

The normal tissue reaction was tested first using the normal tissue antigen and pooled guinea pig antinormal tissue serum. When both of these reagents were at a dilution of 1:2, 4.6 units of complement was fixed. The relatively small amount of fixation occurring when the pooled antiserum was used made it desirable to check the antiserums individually. They were titrated separately with the NTA against varying amounts of complement, and the units of complement fixed for each antiserum determined. The amount of fixation varied from less than the 1.3 units, the lowest amount used in the titrations, to greater than 7.3 units, which was the largest amount employed. Six of the 24 antiserums gave the high titers (7.3 units) and were pooled for use as the antinormal tissue serum. The other 18 samples, whose titers were less than 7.3 units, were discarded. When this pool of guinea pig antinormal tissue serum was titrated against the normal tissue antigen, reactions fixing up to 15 units of complement were obtained (Table XVI). This normal tissue reaction provided a control with which the normal tissue reagent component content of the tumor antigens and antitumor serums could be measured.

TABLE XVI

TITRATION OF POOLED HIGH TITERED GUINEA PIG ANTINORMAL TISSUE SERUM WITH NORMAL TISSUE ANTIGEN

Reaction	Antigen Dil.	Test No.	Antiserum Dilution 1:2	1:4	1:8	1:16
			Units Fixed			
NTA + NTGPS	1:2	(1)	15.2	12.5	8.6	5.1
		(2)	14.9	12.5	8.7	4.8
		(3)	14.8	12.7	8.6	5.3
		Ave.	15.0	12.6	8.6	5.0
	1:4	(1)	8.7	7.2	5.4	4.8
		(2)	9.1	7.5	5.6	4.3
		(3)	9.2	7.6	5.3	4.0
		Ave.	9.0	7.4	5.4	4.2
	1:8	(1)	5.6	4.9	4.8	3.0
		(2)	5.4	5.1	4.5	3.1
		(3)	5.3	4.9	4.4	3.0
		Ave.	5.4	5.0	4.6	3.0
	1:16	(1)	2.9	2.6	2.4	2.1
		(2)	3.1	2.6	2.2	2.2
		(3)	3.0	2.7	1.9	2.2
		Ave.	3.0	2.6	2.2	2.2

Since the antibody formation in the guinea pigs inoculated with normal tissue antigen had shown such a marked variation between individual animals, the testing of pooled samples of the tumor antiserums was not attempted, and tests on the individual antiserums were made. The TA-L1, due to its high anticomplementary activity, was not used as a test antigen. The guinea pig antitumor antigen-L1 serum (TAGPS-L1) and the guinea pig antitumor antigen-L2 serum (TAGPS-L2) were titrated against the TA-L2 in the same manner as was used for the normal tissue materials. Most of the guinea pig antitumor serums failed to show fixation of as much as 2.4 units of complement. However, six of the antiserums from the TAGPS-L1 group and seven from the TAGPS-L2 group gave titers of 23.1 units. These high titered samples were pooled from each group and constituted the antiserums TAGPS-L1 and TAGPS-L2 used in the subsequent tests.

Following the tests on the individual tumor antiserums, the supply of tumor antigen-L2 was practically exhausted, and the tumor antigen-L3 was brought into use. These two antigens (TA-L2 and TA-L3) were titrated against the tumor antiserums (TAGPS-L1 and TAGPS-L2) to determine their relative potency. The amount of fixation occurring in the tests employing the TA-L3 was 20 and 23 units when tested against the L1 and L2 antitumor serums, respectively, which was slightly less than when the TA-L2 was used, which fixed 26 and 28 units. However, the 20 units was sufficient to allow the use of the TA-L3 in the direct tumor tests.

The normal tissue antigen (NTA) and guinea pig antinormal tissue serum (NTGPS) served as control materials with which the reaction of normal tissue components of the tumor materials could be measured. The

specific normal tissue reaction was tested first by preparing two-fold serial dilutions of antigen ranging from 1:2 to 1:32 and dilutions of the guinea pig antinormal tissue serum from 1:2 to 1:16. Three sets of determinations were made in which each dilution of the antigen was tested against each dilution of the antiserum. The results in the duplicate tests were constant and the values for the various reactions averaged. A 1:2 dilution of the antigen and 1:4 dilution of the antiserum fixed 12.5 units and was used as a base line for the determination of the relative amounts of normal tissue reagents in the tumor materials. The normal tissue antibody content of the guinea pig antitumor serums was then determined. The amount of fixation by the normal tissue antigen-guinea pig antitumor serum reaction, when compared to the amount obtained with the normal tissue reagent reaction, indicated the relative concentration of normal tissue antibodies in the guinea pig antitumor serums, with respect to their concentration in the guinea pig antinormal tissue serum. The number of units fixed by the two guinea pig antitumor serums was practically identical, being 12.6 and 12.9 units for the L1 and L2 antiserums, respectively. When compared to the 12.5 units obtained with the guinea pig antinormal tissue serum, it was evident that the normal tissue antibody content in all three of the antiserums was approximately the same.

The next step was the testing of the three antiserums (NTGPS, TAGPS-L1 and TAGPS-L2) against tumor antigen (TA-L3). The concentration of normal tissue antibodies in these antiserums had been shown to be the same. When allowed to react with tumor antigen, the antitumor serums should fix more complement if antitumor cell component antibodies or

antitumor agent antibodies were present. The normal tissue antibody content of the antiserums had been compared when all of them were at a dilution of 1:4; therefore, the same dilution was used and the antiserums tested against varying concentrations of the tumor antigen-L3. As shown in Table XVII, the fixation by the antitumor serums was much greater than with the antinormal tissue serum, being more than twice as great when the antigen was at the 1:8 dilution. This difference was attributed to the reaction of antibodies in the guinea pig antitumor serums which reacted with a specific material in the tumor antigen which was not present in the normal tissue antigen. These reactions were due either to tumor cell constituents and specific antibodies, or tumor agent and its specific antibody. No method was known to differentiate between these reactions. There was the possibility, however, that if chicken antiserums could be obtained which contained only agent antibodies, they could be used to differentiate the portion of the reaction due to the agent, but this would be dependent upon the demonstration of antibodies in chicken serum, which was one of the main objectives of the tests.

The guinea pig antiserums had been used at a dilution of 1:4 for the comparison of their normal tissue antibody content. Other dilutions, namely 1:2 and 1:8, were also prepared and all three dilutions of the guinea pig antitumor and antinormal tissue serums tested against a 1:8 dilution of the tumor antigen-L3. The 1:4 dilutions of the antiserums gave titers which compared favorably with comparable reactions conducted previously. In the reactions with the 1:8 dilutions of the antiserums, there was the expected decrease in fixation with decreased antiserum concentration. However, when the 1:2 dilution of antiserum was involved,

TABLE XVII

TITRATION OF TUMOR ANTIGEN-L3 AGAINST GUINEA PIG ANTITUMOR-L1, ANTITUMOR-L2, AND ANTINORMAL TISSUE SERUMS

Antiserum	Test No.	TA -L3 1:4	1:8	1:16
		Units Fixed		
NTGPS (1:4)	(1)	15.5	10.8	8.1
	(2)	15.2	11.3	8.0
	(3)	15.1	10.9	8.4
	Ave.	15.3	11.0	8.2
TGPS-L1 (1:4)	(1)	>26.0	24.1	13.1
	(2)	>26.0	23.9	13.6
	(3)	>26.0	23.4	13.5
	Ave.	>26.0	23.8	13.4
TGPS-L2 (1:4)	(1)	>26.0	24.2	13.0
	(2)	>26.0	24.1	12.9
	(3)	>26.0	24.6	13.3
	Ave.	>26.0	24.3	13.1

very little increase in the amount of fixation occurred over what had been obtained with the 1:4 dilution. These results showed that when the antigen was at the 1:8 dilution and the antiserum dilution was at 1:4, both reagents were at approximately optimum concentration and the antigen was a limiting factor. Therefore, if chicken antitumor serums were tested in the indirect test, using these concentrations in the direct reaction, any effect they might have on the antigen would be detectable. The standard direct tumor test thus involved TA-L3 at a 1:8 dilution and TAGPS-L1 or TAGPS-L2 at a 1:4 dilution. When normal tissue materials were used in direct tests (for the indirect titration of chicken antiserum), the antigen (NTA) was diluted 1:2 and the antiserum (NTGPS) diluted 1:4.

e. Indirect complement fixation tests on chicken antiserums for the detection of tumor agent antibodies:--The next step was the testing of chicken antiserums indirectly, using the standardized tumor direct reaction. The chicken antiserums produced by the inoculation of the L1 tumor antigen were prepared in dilutions of 1:2 and 1:4 and tested with direct tumor reactions employing tumor antigen-L1 and guinea pig antitumor serums L1 and L2. Normal serums, which had been obtained from the respective birds prior to their inoculation, were also tested. The results of these tests are given in Table XVIII and indicate that three of the four chicken antitumor serums apparently possessed antibodies in low concentration, the highest titer being 2.4 units. The normal serums, however, were without effect. The small values of 0.1, 0.2 and 0.3 units found in many of the control tests were found to be due to irregularities involving the anticomplementary activities of the individual reagents.

TABLE XVIII

INDIRECT TITER OF CHICKEN ANTITUMOR ANTIGEN-L1 SERUMS WHEN TESTED WITH TA-L3 (ANTIGEN) AND TGPS-L1 AND L2 (ANTISERUMS) IN THE DIRECT REACTION

Direct Reaction	Chicken No.	Indirect Titer of Chicken Serum							
		NCS*				TCS -L1*			
		(1)	(2)	(3)	Ave.	(1)	(2)	(3)	Ave.
TA - L3 + TGPS -L1	K1412J_2	0.2	0.2	0.3	0.2	1.2	1.3	1.5	1.3
	K1401R_3	0.1	0.3	0.1	0.2	0.1	0.4	0.3	0.3
	K1301W	0.0	0.1	0.1	0.1	2.2	2.4	2.5	2.4
	K1310X	0.1	0.2	0.0	0.1	1.8	1.7	1.7	1.7
TA - L3 + TGPS -L2	K1412J_2	0.0	0.1	0.1	0.1	1.3	1.5	1.6	1.5
	K1401R_3	0.1	0.2	0.2	0.2	0.3	0.4	0.3	0.3
	K1301W	0.2	0.3	0.3	0.3	1.9	1.9	2.0	1.9
	K1310X	0.1	0.1	0.0	0.1	1.7	1.9	1.8	1.8

*Chicken serum used at dilution 1:2

The direct reaction was standard, and since the titers of the chicken antiserums were the only values of importance in these tests, they were the only ones used in the evaluation of the indirect test results.

It will be observed in Table XVIII that the type of guinea pig antiserum employed had no effect on the chicken antiserum titer. This was expected since the actual reaction being measured involved the chicken antiserum and the antigen, while the guinea pig antiserum was, in reality, a part of the indicator system. The nature of the reaction by the chicken antiserums, however, was not known. The tumor antigen contained agent, normal tissue cell components and tumor cell components. Any of these might have stimulated antibody formation in the chicken and been responsible for the indirect titers observed. If normal tissue antibodies were present and responsible for the titers obtained, the substitution of normal tissue antigen (NTA) for the tumor antigen (TA-L3) in the direct test should give positive titers for the chicken antitumor serum of approximately the same magnitude as was obtained with the tumor antigen. This was done, and as shown in Table XIX, titers almost identical to those found with the tumor antigen were obtained. Therefore, the antibodies in the chicken antitumor serums apparently had been produced by normal tissue components of the tumor antigen, and antiserums from chickens which had been inoculated with normal tissue antigen should give similar reactions. This was found to be the case when chicken antinormal tissue serums were tested using the same direct reactions as employed in the testing of the chicken antitumor serums. The results of these tests are shown in Table XX. Slightly higher chicken antinormal tissue serum titers were obtained when normal tissue antigen was combined

TABLE XIX

INDIRECT TITER OF CHICKEN ANTITUMOR ANTIGEN-L1 SERUMS WHEN TESTED WITH NTA (ANTIGEN) AND TGPS-L1 AND L2 (ANTISERUMS) IN THE DIRECT REACTION

Direct Reaction	Chicken No.	Indirect Titer of Chicken Serum NCS* (1)	(2)	(3)	Ave.	TCS -L1* (1)	(2)	(3)	Ave.
NTA + TGPS -L1	K1412J_2	0.0	0.1	0.1	0.1	1.5	1.4	1.4	1.4
	K1401R_3	0.1	0.2	0.1	0.1	0.1	0.3	0.0	0.2
	K1301W	0.1	0.0	0.0	0.0	1.7	1.9	1.8	1.8
	K1310X	0.3	0.2	0.3	0.3	1.7	1.6	1.6	1.6
NTA + TGPS -L2	K1412J_2	0.1	0.2	0.2	0.2	1.3	1.4	1.4	1.4
	K1401R_3	0.0	0.1	0.0	0.0	0.1	0.2	0.2	0.2
	K1301W	0.3	0.1	0.2	0.2	2.4	2.1	2.0	2.2
	K1310X	0.1	0.2	0.1	0.1	1.9	1.8	1.8	1.8

*Chicken serums used at dilution 1:2

TABLE XX

INDIRECT TITER OF CHICKEN ANTINORMAL TISSUE SERUMS WHEN TESTED WITH NORMAL TISSUE AND TUMOR ANTIGENS AND GUINEA PIG ANTINORMAL TISSUE AND ANTITUMOR SERUMS IN THE DIRECT REACTION

Direct Reaction	Chicken No.	Indirect Titer of NTCS* (1)	(2)	(3)	Ave.
		Units			
TA -L3 + TGPS -L1	K1310G_2	1.4	1.7	1.8	1.6
	K1310E_2	2.4	2.8	2.8	2.7
	K1310D_2	0.5	0.3	0.5	0.4
	K1413H_2	1.3	1.5	1.2	1.3
NTA + TGPS -L1	K1310G_2	2.4	2.4	2.2	2.3
	K1310E_2	3.0	3.2	2.9	3.1
	K1310D_2	0.7	1.0	1.0	0.9
	K1413H_2	2.3	1.9	2.0	2.1
TA - L3 + NTGPS	K1310G_2	1.6	1.3	1.3	1.4
	K1310E_2	1.9	2.1	1.8	1.9
	K1310D_2	0.0	0.3	0.0	0.1
	K1413H_2	1.7	1.7	1.8	1.7
NTA + NTGPS	K1310G_2	2.6	2.8	2.6	2.7
	K1310E_2	3.1	3.6	3.2	3.3
	K1310D_2	0.2	0.4	0.4	0.3
	K1413H_2	2.2	2.0	2.6	2.3

*NTCS used at dilution 1:2

with either guinea pig antinormal tissue or antitumor antigen-L1 serums in the direct reaction, but the differences were not sufficient to indicate any specificity of the chicken anti normal tissue serums. The supply of guinea pig antitumor serums was quite limited and since no difference had been noted between them in any of the reactions, only one of them - the guinea pig antitumor antigen-L1 serum - was used in these tests.

It was evident that the antibodies demonstrated to be present in the chicken antitumor antigen-L1 serums were specific for tissue materials but not the tumor agent. The marked reaction of the guinea pig antitumor serums with tumor antigen, as compared to their reaction with normal tissue antigen in direct reactions, had indicated the presence of specific materials other than normal tissue, but the chicken antiserums apparently reacted with only the normal tissue portion. Therefore, it was necessary to obtain chicken antiserum which, when tested against tumor antigen, would cause more fixation than when tested against normal tissue antigen. By using the direct reactions TA-L3 + TGPS-L1, and NTA + TGPS-L1 for the testing of chicken anti tumor serums, the presence of more fixation by the former reaction would indicate the presence of agent antibodies in the chicken serum. The subsequent tests, therefore, employed these two direct reactions, the former being referred to as the tumor direct reaction and the latter as the normal tissue direct reaction.

Ducks had been inoculated with tumor and normal tissue antigens (TA-L1 and NTA) and with normal and infected chicken serums (NCS and ICS). Duck antiserums act similar to the chicken antiserums in that they produce no fixation when used in direct tests. Duck antinormal tissue and antitumor serums were tested by the indirect technique. The NTDS and

NCSDS acted as controls, and the TDS-L1 and ICSDS served as potential sources of tumor agent antibody. As can be seen in Table XXI , antibodies had been produced in all the ducks inoculated with the normal tissue or tumor material except duck number 2, which was in the group that had received the tumor antigen-L1. The titers of the duck antitumor and antinormal tissue serums were approximately the same when tested with either the tumor or normal tissue direct reactions, indicating again that only normal tissue antibodies were present. The duck antinormal and anti-infected chicken serum serums failed to show any reaction indicating the absence of antibody formation.

As was mentioned previously, infected chicken serum might contain antibodies specific for the agent. This serum, having been secured from birds showing visceral tumors, had been used as a potential source of agent in previous experiments. However, it was not known if birds, when showing advanced tumor involvement, produced specific tumor agent antibodies. When the serums of infected birds were pooled for the production of the ICS antigen, samples of these having the largest volumes had been saved and refrozen. These serums were removed from storage and used as chicken antitumor serums in indirect tests. As will be seen in Table XXII, none of these serums exhibited positive titers when tested with the tumor direct reaction, which indicated the absence of either tissue or agent antibodies. The lack of any reaction with the tumor direct reaction made it unnecessary to conduct tests on them with normal tissue direct reaction.

Chickens were inoculated with tumor antigens prepared in various ways in an attempt to stimulate agent antibody production. The various

TABLE XXI

INDIRECT TITER OF DUCK ANTISERUMS WHEN TESTED WITH NORMAL TISSUE AND TUMOR ANTIGENS AND GUINEA PIG ANTITUMOR-L1 SERUM IN THE DIRECT REACTION

Duck Antiserum	Duck No.	Test No.	Direct Reaction TA -L3 + TGPS -L1*	Direct Reaction NTA + TGPS -L1*
			Units	
NTDS (1:2)	5	(1)	4.5	4.6
		(2)	4.1	4.7
		Ave.	4.3	4.6
	6	(1)	2.8	3.3
		(2)	2.6	2.9
		Ave.	2.7	3.1
TDS -L1 (1:2)	1	(1)	3.7	4.5
		(2)	4.1	4.4
		Ave.	3.9	4.4
	2	(1)	0.2	0.1
		(2)	0.6	0.0
		Ave.	0.4	0.1
	3	(1)	2.1	1.5
		(2)	2.0	1.8
		Ave.	2.0	1.6
	4	(1)	3.9	4.1
		(2)	4.3	4.7
		Ave.	4.1	4.4
NCSDS (1:2)	74	(1)	0.1	0.0
		(2)	0.1	0.4
		Ave.	0.1	0.2
	75	(1)	0.3	0.1
		(2)	0.1	0.0
		Ave.	0.2	0.0
ICSDS (1:2)	71	(1)	0.1	0.2
		(2)	0.0	0.0
		Ave.	0.0	0.1
	73	(1)	0.5	0.1
		(2)	0.4	0.0
		Ave.	0.4	0.0
NDS (1:2)	Pool	(1)	0.0	0.0
		(2)	0.1	0.2
		Ave.	0.0	0.1

* Direct reactions used to test duck antiserums

TABLE XXII

INDIRECT TITERS OF INDIVIDUAL INFECTED CHICKEN SERUMS WHEN TESTED WITH TUMOR ANTIGEN-L3 AND GUINEA PIG ANTITUMOR-L1 SERUM IN THE DIRECT REACTION

	Chicken Serum (ICS) dilution		
Chicken No.	1:1	1:2	1:4
K2P25	0.0	0.1	0.0
K1517H	0.1	0.1	0.0
K1401E	0.0	0.0	0.0
K1516H	0.2	0.1	0.3
K1463P	0.2	0.0	0.3
K1511O	0.0	0.0	0.2
K1508Z	0.2	0.1	0.2
K1406R	0.0	0.0	0.0
K1511N	0.0	0.1	0.1
K1507Z	0.2	0.1	0.1
$K1519D_2$	0.3	0.2	0.3
$K1504M_2$	0.0	0.1	0.0
$K1414A_3$	0.1	0.0	0.0
$K1521C_3$	0.0	0.1	0.0

chicken antitumor serums were tested with the tumor and normal tissue direct reactions. The titers obtained for these antiserums, as shown in Table XXIII, varied from 0 to 4.1 units. As had been the case in previous tests, the titers were approximately the same when the normal tissue direct reaction was used as when the tumor direct reaction was employed. It was, therefore, concluded that chickens had produced antibodies specific for tissue material but had not produced detectable tumor agent antibodies. No method was known which would be of value in decreasing the normal tissue and tumor tissue materials of the antigens other than those employed. The development of a complement fixation test for the detection of either the agent of avian visceral lymphomatosis or specific antibodies will depend on the development of techniques for the purification of the agent. The centrifugation techniques now available do not allow sufficient separation of the agent and these tissue materials for the detection of an agent stimulated reaction.

The removal of the normal tissue antibodies from the tumor guinea pig antiserum was considered as a possible method for the elimination of the normal tissue reaction which interfered with the determination of the presence of a tumor agent-agent antibody reaction. By removing the normal tissue antibodies from the guinea pig antitumor serum, no fixation of complement by a normal tissue reaction would occur, even though there were normal tissue materials present in the antigen.

A suspension of normal tissue material similar to the normal tissue antigen could not be used for the adsorption of the normal tissue antibodies from guinea pig antiserums since all of the tissue components could not be removed following the adsorption, and the portion remaining

TABLE XXIII

INDIRECT TITER OF VARIOUS CHICKEN ANTITUMOR SERUMS WHEN TESTED WITH NORMAL TISSUE AND TUMOR DIRECT REACTIONS

Chicken Antiserum	Chicken No.	Direct Reaction TA -L3 + TGPS -L2 Units	Direct Reaction NTA + TGPS -L2 Units
TA -PT4	K1466C	2.1	2.4
	K1513I	3.7	4.1
	K2P95	1.7	2.0
TA -L4A	K1503O	0.2	0.0
	K2P100	1.9	2.0
	K5P42	0.3	0.4
TA -L4B	K2P93	1.4	1.9
	K5P46	0.1	0.3
TA -L4C	K2P89	2.6	3.0
	K2P97	0.7	1.2
	K5P49	3.4	2.9
TA -L5A	K2P99	1.7	1.9
	K2P96	2.1	1.7
	K1455Q	1.4	1.1
TA -L5B	K5P52	2.6	2.2
	K1473Y	0.4	0.0
	K1473Z	3.6	3.1
TA -L5C	K2P90	0.7	1.1
	$K1414E_2$	2.1	1.7
	K2P72	0.2	0.3
TA -L7A	K2P99	3.6	2.9
	$K1455Q_2$	1.6	2.0
	K2P98	2.4	2.3
TA -L7B	K2P42	0.7	0.5
	K1473Y	1.9	2.2
	$K1414D_2$	0.2	0.1
TA -L9	$K1473L_2$	1.6	1.9
	$K1473O_2$	0.3	0.1
	$K1516L_2$	2.7	3.0
	K620Q	3.8	3.4
	$K669L_2$	0.7	0.6
TA -Ly1	K258K	1.5	1.9
	K262B	0.2	0.0
	$K669X_2$	0.1	0.2
	K1466M	2.7	3.0
	$K1511H_2$	3.6	3.4
	K350S	1.2	0.9
	K620B	3.8	3.5
	$K669C_3$	0.2	0.0

would combine with normal tissue antibodies, forming a complete normal tissue system which would fix complement. Chicken red blood cells provided a source of normal chicken tissue which would eliminate this difficulty. The red blood cells from apparently normal chickens of approximately 500 days of age were used. The chicken red blood cells could be washed and measured and after mixing with the antiserum, could be removed in their entirety, leaving the antiserum unchanged except for the normal tissue materials that had been adsorbed on the red cells. The guinea pig anti normal tissue and anti tumor antigen-L1 serums were mixed with 10 volumes of packed chicken red blood cells. The cells were suspended in the serums by shaking and allowed to incubate for 30 minutes at 37° C. This was repeated a total of 10 times. Agglutination of the cells was noted in the first five or six adsorptions but became increasingly less until none was observed after the seventh adsorption. Volumes of 1L milliliters for the guinea pig antinormal tissue serum and 16 milliliters for the guinea pig antitumor antigen-L1 serum were recovered. These were first used in direct tests with normal tissue antigen and tumor antigen-L3. When the titers of the adsorbed guinea pig antiserums were compared to the titers obtained before adsorption, it was evident, as shown in Table XXIV, that considerable normal tissue antibody had been removed, especially from the guinea pig antitumor serum. The reaction of the guinea pig antitumor serum, remaining after adsorption, might be due in a greater proportion to tumor agent-agent antibody reaction, which would increase its usefulness when testing chicken serum for its agent antibody content. The only method available to determine if this were true was the testing of chicken antiserum believed to contain agent antibodies.

TABLE XXIV

TITER OF GUINEA PIG ANTINORMAL TISSUE AND ANTITUMOR-L1 SERUMS BEFORE AND AFTER ADSORPTION WITH CHICKEN RED BLOOD CELLS

Treatment	Antigen	Dilution	Antiserum: NTGPS 1:2	NTGPS 1:4	NTGPS 1:8	TGPS -L1 1:2	TGPS -L1 1:4	TGPS -L1 1:8
Before Adsorption	TA -L3	1:8	11.7	10.4	6.2	26.1	24.1	15.4
	NTA	1:2	15.2	12.6	8.6	15.2	12.4	8.8
After Adsorption	TA -L3	1:8	7.8	5.9	4.6	14.5	11.2	8.3
	NTA	1:2	8.2	6.6	5.4	7.1	5.3	4.0

The chicken antitumor serums which had given the highest titers when tested with the unadsorbed materials were again tested, employing the adsorbed guinea pig antitumor serum in the tumor and normal tissue direct reactions. As shown in Table XXV, little or no change in the titer of the chicken serums could be noted when compared to the titers obtained when the unadsorbed antiserum had been used in the direct reactions.

The reduction of the amount of fixation in the direct reactions indicated that the concentration of normal tissue antibodies was reduced by adsorption with chicken red blood cells, but their removal was not complete. The similar titers of the chicken antiserums with both the normal tissue and tumor direct reactions conclusively demonstrated that the antibodies in the chicken antitumor serums were specific for normal tissue materials and not the tumor agent. This further emphasized the absence of tumor agent antibodies in the chicken antitumor serums, and the necessity of obtaining more purified reagents for use in the production of antiserums free of normal tissue contamination.

TABLE XXV

TITERS OF VARIOUS CHICKEN ANTITUMOR SERUMS WHEN ADSORBED GUINEA PIG ANTITUMOR SERUM USED IN DIRECT REACTIONS

Chicken Antiserum	Chicken No.	Direct Reaction	
		TA -L3 + TGPS -L1*	NTA + TGPS -L1*
		Units	
TA -PT4	K1466C	1.7	1.8
	K1513I	3.1	3.0
TA -L4A	K2P100	1.4	1.1
TA -L4B	K2P93	0.7	1.0
TA -L4C	K2P89	1.8	2.0
	K5P49	2.1	2.6
TA -L5A	K2P99	1.6	1.4
	K2P96	1.3	1.6
TA -L5B	K5P52	2.4	2.6
	K1473Z	3.3	2.9
TA -L5C	$K1414E_2$	1.2	0.8
TA -L7A	K2P99	2.1	2.4
	K2P98	1.1	1.4
TA -L7B	K1473Y	1.8	1.7
TA -L9	$K1473L_2$	1.2	1.8
	$K1516L_2$	3.0	2.4
	K620 0	3.0	3.4
TA -Ly1	K258K	0.9	1.2
	K1466M	1.7	2.1
	$K1511H_2$	2.8	2.4
	K620B	2.6	3.2

* Direct reaction used for testing chicken antitumor serums

VIII. SUMMARY

Avian visceral lymphomatosis, a malignant disease of chickens, is a major economic problem to the poultry industry. No effective method for the control of this disease is known. Genetic resistance and isolation procedures may influence the incidence of infection but are not practical for large scale application. With the isolation of an infectious agent (Furth and Breedis, 1933) and subsequent developments in techniques applicable to its characterization, the possibility of the development of a test was considered. The complement fixation test was selected for use in an attempt to develop such a test.

The complement fixation test, as used for routine laboratory diagnosis of various diseases, cannot be adapted to the testing of chicken serum. Antibodies produced in the chicken will combine with specific antigen but the resulting complex is not capable of fixing complement (Rice, 1948b). The indirect complement fixation technique must be employed. Attempts were made to conduct tests according to the techniques described by Rice (1946) and Mayer *et al.* (1947), in which the complement is measured in terms of units which are calculated by the use of the hemolytic slope. A consistent value for the slope could not be obtained for identical complement titrations. The slope was also calculated for a group of titrations when influenza antigen or rabbit anti-influenza serum was present, and a fourth group when both of these reagents were present. When either the antigen or antiserum was present, slope values, considerably above the 0.20 given by Mayer *et al.* (1946b) and Rice (1942) as the optimal value, were obtained. When both the

antigen and antiserum were present, the average of the determinations was 0.21, but varied from 0.15 to 0.27.

The inconsistency of the slope values, which were used for the calculation of the units of fixation, necessitated the development of a technique in which the slope calculation was not necessary. The determination of the titer of complement consisted of testing varying amounts of the complement and determining the amount that would be needed to produce exactly 50 percent hemolysis. This could be done quite easily by plotting the volume of complement against the percent of hemolysis. The hemolysis was measured with an electrophotometer and since the log scale readings were proportional to the hemolysis, the volume of complement could be plotted directly against these readings. The amount of complement necessary to give 50 percent hemolysis when any reaction occurred could be determined in a similar manner. The actual plotting of these values was eliminated by the preparation of a table of proportionality values from which the proportionate distance between any two volumes of complement, where the amount necessary for 50 percent hemolysis fell, could be determined from the electrophotometer readings. By determining the amount of complement necessary for 50 percent hemolysis when a given reaction was present and dividing it by the complement unit value, the number of units fixed by the reaction could be calculated.

Influenza, normal tissue, and tumor antigens were used in the various tests. These antigens were used for antiserum production in rabbits, guinea pigs, chickens and ducks. Normal and infected chicken serums were also used as antigens in tests and for inoculation.

Influenza antigen and antiserums were used to standardize the test procedure. Rabbit anti-influenza serum was used in the original tests and a direct test was standardized. When chicken influenza antiserum was tested by the indirect technique, the amount of fixation was increased, instead of decreased, over what had occurred in the direct reaction, as should have been the case if influenza antibodies were present in the chicken antiserum. This was found to be due to the reaction of heterophile antigen in the chicken serum with specific antibodies in the rabbit antiserum. These antibodies had been produced by heterophile antigen in the influenza antigen material and had originated from the embryos used for the influenza virus propagation. Guinea pigs were found not to produce the heterophile antibodies, and the standardization of the technique was completed by the determination of the optimal times and temperatures of incubation and the order in which the reagents were to be added.

The first tests on tumor materials were made using infected chicken serum as an antigen against guinea pig anti-infected chicken serum serum. No reaction between these materials could be detected and liver tumor extract was substituted as the antigen for the infected chicken serum. Slight reactions were noted but were not specific since similar reactions could be obtained with normal tissue antigen. Guinea pig antiserums, produced with normal tissue and tumor antigens, were tested using normal and infected chicken serums as antigens, and again slight reactions were noted but were found to be due to normal tissue materials. It was, therefore, concluded that the concentration of the agent in infected chicken serum was not sufficient to stimulate antibody formation in the guinea pig or to act as specific antigen in the presence of tumor guinea pig antiserum.

The standardization of a direct reaction with which the chicken antitumor serums could be tested had to take into consideration the presence of normal tissue reactions. Guinea pig antitumor serum was tested against normal tissue antigen and by comparing its titer with the titer of guinea pig antinormal tissue serum, the normal tissue antibody content of the guinea pig antitumor serum was determined. Tumor antigen was then used for testing these antiserums, and considerably greater reaction was noted with the guinea pig antitumor serum than with the guinea pig antinormal tissue serum. This indicated the presence of a specific reaction between the tumor reagents other than that which could be accounted for by the normal tissue materials. This specific reaction was considered due to one of two systems -- either the agent and its antibody, or tumor cell components and antibodies specific for them.

Chicken antitumor serums were tested with a standardized tumor direct reaction employing tumor antigen and guinea pig antitumor serum. The chicken serum titers, although quite low, showed that antibodies were present and had inactivated a portion of the antigen. Normal tissue antigen was substituted for the tumor antigen in the direct reaction and the chicken antiserums again tested. The indirect titers obtained were almost identical with those found when the tumor antigen had been used. Thus, the antibodies in the tumor chicken antiserum had apparently been produced as a result of normal tissue stimulation. A normal tissue reaction had not been expected. When guinea pigs were inoculated with tumor antigen, antibodies other than the normal tissue type had been produced, but with the inoculation of chickens, little or no normal tissue reaction had been expected. The inoculation of normal chicken tissue into its

homologous host was considered much less likely to stimulate antibody production than would the presence of tumor cell components or tumor agent which would be considered foreign material. As a check on this finding, antiserums from chickens which had been inoculated with normal tissue antigen were titrated. Approximately the same titers were obtained for the chicken antinormal tissue serums with either the tumor or normal tissue direct reaction, and were of approximately the same magnitude as the titers of the chicken anti tumor serums. It was, therefore, concluded that the lymphomatosis agent in the tumor antigen had not stimulated antibody production in the chicken.

Ducks were inoculated with tumor and normal tissue antigen, and infected and normal chicken serums. The tumor and normal tissue antigens stimulated antibody formation, but as had been found with the chicken antiserums, a reaction with tumor antigen was accompanied by a similar reaction with normal tissue antigen. The duck anti-infected and anti-normal chicken serum serums failed to exhibit any reaction, indicating the complete absence of antibody formation.

The infected chicken serum, previously used as an antigen, was tested by the indirect method. This serum could have reacted in either of two ways. If antibodies had been formed during the advanced stages of tumor development, they might inhibit the reaction of the agent with guinea pig antibodies. The second reaction could arise if agent were present which would react with antibodies in the guinea pig antiserum, and in this way, also inhibit the reaction of the antigen and antibody of the direct test. This second possibility was eliminated when the infected chicken serum had been used as an antigen in direct tests;

therefore, if an indirect titer were obtained for the infected chicken serum, it would have been due to the presence of agent antibodies. This was not the case, however, since no antibodies could be detected when it was tested in the indirect reaction.

A group of tumor antigens was prepared using various centrifugation procedures in an attempt to procude a suspension of the agent which would stimulate specific antibody formation when inoculated into chickens. The chicken antitumor serums prepared with them exhibited varying degrees of reactivity when tested with direct reactions employing tumor antigen. The titers ranged from 0.1 to 3.8 units, but as had been found in all previous tests of this type, a reaction with the tumor antigen was accompanied by a similar reaction against normal tissue antigen. Adsorption of the guinea pig antitumor serum with normal chicken red blood cells removed a portion of the normal tissue antibodies but did not facilitate the detection of agent antibodies in the chicken antitumor serums. From these findings, it was concluded that the chickens had produced normal tissue antibodies but none which were specific for the agent of visceral lymphomatosis, and the development of a complement fixation test is dependent upon the elimination of the normal tissue materials.

CONCLUSIONS

1. The hemolytic slope value shows wide variation and changes in the presence of various test reagents, making it unsuitable for use in the calculation of the amounts of complement fixed.

2. Complement fixation, determined graphically in terms of milliliters of undiluted complement, can then be converted to units and eliminate the use of slope calculations.

3. When the test antigen contains chicken tissue material, rabbits can not be used for the production of complement fixing antiserum, due to the formation of heterophile antibodies which react with the heterophile antigen of the chicken serum. Guinea pigs do not form this antibody.

4. Infected chicken serum does not stimulate the production of agent antibody formation in guinea pigs, and fails to give a specific reaction when tested with tumor guinea pig antiserum.

5. Tumor antigen stimulates the formation of antibodies in guinea pigs which are distinguishable from normal chicken tissue antibodies, but are apparently due to the presence of tumor cell components and not the tumor agent.

6. The inoculation of chickens with tumor antigens stimulates the formation of antibodies which react similarly with tumor antigen or normal tissue antigen, and there is no evidence of the production of tumor agent antibodies.

7. The utilization of the complement fixation test in the detection of the agent of avian visceral lymphomatosis will depend upon the development of techniques for the increased purification and concentration of the agent.

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