A STUDY ON

SOME ASPECTS OF LEPTOSPIRAL ANTIGENS AND ANTIBODIES

By

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ABSTRACT

The leptospirae have been classified principally on the basis of antigenic relationships since the usual biochemical and morphological methods are of little or no value. Qualitative serological differentiation based upon antigenic characteristics of species has proved to be of tremendous aid, but even this method did not show sharp distinction among serotypes and considerable serologic cross agglutination is observed. Quantitative serological studies were conducted in the hope that this method would permit recognition of distinct antigenic differences among these organisms and thereby allow serologic classification without ambiguity.

Qualitative and quantitative examinations of L. icterohaemorrhagiae AB, L. canicola and L. pomona were conducted to ascertain the antigenic interrelationships. Before quantitative studies of the leptospiral antigen antibody system could be made, certain preliminary tests and standards had to be completed. Then, a study was conducted to determine the time, temperature and ratio of antigen to antibody necessary for complete adsorption of antibodies from antisera produced in rabbits. By using the proportion of antigen to antibody which agglutinated most rapidly in a series of mixtures, it was found that incubation at 37°C for 2 hours and 1°C over night resulted

in complete adsorption of the antiserum.

Reciprocal cross agglutinin adsorption tests indicated that the three serotypes contain one antigen in common and that L. icterohaemorrhagiae AB contains two other antigens, one common to L. canicola and the other identical with $\overset{a}{\wedge}$L. pomona antigen.

Quantitative estimations of the antibody nitrogen in antisera were made by two separate methods. With whole serum the amount of N in the antigen and the specific precipitate was determined by direct micro-Kjeldahl analysis. The difference between the two values equals the milligrams of antibody nitrogen precipitated. With purified antibody solutions the amount of nitrogen in the supernatant fluid before and following adsorption was measured by the phenol reagent method and the difference in nitrogen was converted by an albumin standard to micro-Kjeldahl nitrogen. In the latter procedure the nitrogen of the antigen was obtained by converting optical density of the cell phenol reagent solution, by reference to a standard curve, into micro-Kjeldahl nitrogen.

A quantitative measure of the homologous antibody content of a serum and the amount of this antibody which combined with the two heterologous serotypes indicated that L. pomona is more similar antigenically to L. ictero haemorrhagiae AB than to L. canicola. The latter two species, however, are more closely related antigenically

than L. pomona and either of the other two serotypes. The extent of cross reaction indicates a constant reciprocal antigenic relationship among the three serotypes.

A quantitative study of the combining ratio of antigen N to antibody N showed that the amount of antibody N precipitated was directly proportional to the amount of antigen N added. However, the combining ratios observed in the antibody excess zone was not described by the mathematical expressions derived for similar antigen antibody systems.

Since it was determined that formalin caused some change in the leptospiral cell which was observable by serological methods, relationships derived from these experiments with formalized antigens cannot be applied to these organisms in their natural state without qualification. A study of the agglutination test showed that the routine procedure of testing antisera pro duced significant error in the estimation of antibody. This work indicated the leptospiral cells contain more than one antigenic component and probably contain several antigens. Part of the cross reaction of these serotypes also might be explained by the presence of chemically related antigens. The results of qualitative examinations coupled with the reports of other workers, furnished sufficient information for the construction of probable antigenic structures for the serotypes studied as follows:

L. icterohaemorrhagiae AB a (x, y), b, c . .

L. icterohaemorrhagiae A a (x, y), b

L. canicola	a (x), c, d . . .
L. pomona	b, c, e . . . . .

By quantitative serological methods, it is possible to obtain confirmation of qualitative results and aid in interpretation of them. In this way a clearer insight into the antigenic interrelationships of these serotypes has been obtained. The study of many more serotypes by this combination of methods might reveal further interrelationships and eventually allow the construction of complete antigenic compositions for many of the members of the genus.

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INTRODUCTION

Leptospiral species have been characterized largely by interpretation of qualitative serological reactions. Cross agglutinin adsorption tests have proven useful in differentiation of leptospiral serotypes (72, 70). Sharp distinction among species or serotypes has not been achieved by this method, rather considerable "overlapping" of serologic groupings has been demonstrated (71).

Antigenic differences elucidated by quantitative serological techniques have aided in the characterization of many bacteria (34, 38, 65), however little use has been made of quantitative methods in investigations of the leptospirae. It was thought that a precise measure of the antibody reactive with the homologous and heterologous serotypes might point out characteristic antigenic differences in these organisms and present a clearer picture of the antigenic composition of leptospirae.

The serological differentiation of strains of pathogenic leptospirae is not of academic interest only. This differentiation has proved to be of considerable practical value with regard to epidemiology and ecology, immunotherapy and prophylaxis and in forensic matters (70). L. icterohaemorrhagiae (Weil's disease), L. canicola (canicola fever) and L. pomona (swineherd's disease) cause serious infections in man and animals (74, 47, 1, 55) and consider-

able serologic cross reaction is observed among these serotypes (73, 3, 44, 70). Qualitative and quantitative examinations of these serotypes were conducted to ascertain the antigenic interrelationships.

First, a study was made to determine the optimum time, temperature and antigen antibody ratio for the complete removal of leptospiral antibodies from antisera produced in rabbits. Reciprocal cross adsorptions were conducted to determine qualitative relationships. A quantitative microdetermination of agglutinins in whole serum was made by determining the difference between the N content of the bacterial suspension before and after adsorption. Agglutinin content was determined by measuring the nitrogen in purified antibody solutions before and following specific agglutination. The homologous antibody content of a serum and the amount of this antibody which combines with two heterologous serotypes was studied. Based on qualitative and quantitative data, a relative antigenic structure was proposed for L. pomona, L. icterohaemorrhagiae AB and L. canicola.

LITERATURE REVIEW

Early in the investigation of leptospirae it became apparent that morphological characteristics, biochemical reactions, growth requirements, the clinical picture encountered, the geographical distribution of the organism, the species of the animal host or reservoir, the manner of acquiring infection and the pathogenicity of a given Leptospira for a laboratory animal are not sufficiently different among the leptospiral strains to be used as a basis of characterization. But the specific antigenic characteristics of these organisms as demonstrated by agglutination-lysis reactions proved to be constant enough for differentiation. Taxonomic classification then became possible only through serological procedures.

Wolff and Broom (72) asserted that a classification can be based on the principle that the agglutination-lysis test reveals the stable and specific antigenic characteristics of the members of the genus Leptospira. Wolff (70) then divided the strains of this genus into serogroups and serotypes by use of the cross agglutination-lysis reactions and cross adsorption tests.

At first, antigenic analysis of leptospiral isolates divided the organisms into antigenically well defined groups. Antigenic classification became more complicated, however, as more strains were isolated with serological

patterns which showed "overlapping" or deviations from the established serogroups. It became more difficult to fit these serologically aberrant strains into a fixed serologic scheme (71).

New attempts were made to ascertain the antigenic components of leptospirae in order to establish a better scheme of classification and to develop a simple serological diagnostic procedure which would identify the infecting organism.

Noguchi (50) in 1920, Bessemans et al. (7) in 1928, Gaehtgens (27, 28) in 1933 and 1950, Pot et al. (53) in 1936, Brown et al. (12) in 1939, York (75) in 1952, and Stoenner (64) in 1953 prepared complement fixing antigens from leptospirae using suspensions of intact organisms inactivated by formaldehyde or phenol. Randall et al. (54) in 1949 prepared complement fixing antigens of broad specificity from suspensions of organisms disrupted by sonic vibration.

Hindle and White (39) in 1933 described a water soluble polysaccharide, extracted by acetone, from Leptospira biflexa which was highly reactive in the precipitin test with homologous antisera stimulated by whole organisms. However this antigen did not cross react with antisera against any of the other common leptospirae.

Carlinfanti (13) in 1941 described a "lipoid" antigen obtained by ethanol extraction of washed, dried leptospirae

Boerner et al. (8) in 1941 discovered in the supernatant fluids of 7 day old cultures, heated at 100°C for 2 hours, type specific complement fixing antigens. Ezell et al. (25) in 1952 reported the existence of type specific complement fixing antigens in the cell free culture liquor, which are ethanol soluble, resist exposure at 100°C for 30 minutes and are probably non protein in nature. Pike et al. (52) in 1953 compared soluble complement fixing antigens prepared by three different methods (25, 58, 75) which were type specific and considered to be identical. Terzin (67) in 1956 described soluble type specific complement fixing antigens present in the supernatant fluids of boiled cultures which resemble those reported by Ezell et al. and Boerner et al. (8).

Schneider (58) in 1953 and in 1954 (60, 59) made several types of extracts which in the complement fixation test were reactive with homologous antisera prepared against whole organisms and cross reacted to a lesser extent with antisera prepared against the cells of other leptospiral serotypes.

Hashimoto (31) in 1954 sensitized sheep erythrocytes for hemolysis by antibody, by pretreating them with an extract obtained by boiling concentrated suspensions of leptospirae. The sensitizing antigen was found to be common to the three serotypes studied.

Chang and McComb (14) in 1954 described an erythrocyte

sensitizing substance (ESS) prepared using bile salt alcohol treatment of leptospirae. This substance exhibited a uniform level of reactivity in hemagglutination tests with rabbit or human antisera against all leptospiral serotypes tested. Cox (18) in 1955 showed that ESS was capable of sensitizing erythrocytes for lysis.

Schneider (61) in 1955 isolated in two cell free preparations virtually all of the leptospiral cell's antigenic principle which are reactive in the complement fixation test with hyperimmune rabbit serum. Two serotypes were studied. The aqueous extract contained two immunologically distinct antigens, namely a genus specific complement fixing principle and a serogroup specific agglutinogen. The alcoholic extract reacts as a partial antigen or haptene in rabbits. The antibodies of homologous hyperimmune serum react well in the complement fixation test with the latter antigen and are sharply serotype specific. The aqueous cell free extract, compared to the viable whole leptospiral organism, possesses the property of stimulating the formation of a specific serogroup agglutinin without a detectable genus reactive agglutinin. An obvious application of the property in the classification of leptospirae is that strains can be separated into serogroups without recourse to absorption studies.

Rothstein and Hiatt (56) in 1956 prepared serologically reactive extracts from 25 leptospiral cultures, representing

20 different serotypes and studied their immunological, chemical and physical properties. An ethanol extract of leptospiral cells was divided into two portions on the basis of solubility in distilled water. The soluble portion when introduced into rabbits elicited genus specific precipitins and type specific agglutinins indicating the presence of two antigenic components. By absorption with the homologous organism the agglutinins could be removed from the antiserum. Rothstein and Hiatt (56) postulate from these findings that leptospirae contain two major antigenic components: a P antigen, which is a peripheral type specific principle, and an S antigen, which is a somatic, genus specific principle. The S antigen appears to be a lipo-polysaccharide; the chemical nature of the P antigen was not determined.

Schubert et al. (62) in 1956 compared whole cultures, supernatant fluid of whole cultures, buffered saline suspended leptospirae and ultrasonic vibrated organisms of seven strains as complement fixing antigens and found that the organisms suspended in barbital saline gave best results for detecting leptospiral antibodies.

Muraschi et al. (49) in 1956 reported that ethylene glycol extracts from formalized whole organisms yield a colloidal aqueous complement fixing antigen solution which is sensitive in the detection of leptospiral antibodies.

Cox (19) in 1957 prepared genus specific erythrocyte sensitizing antigens (similar to Chang's ESS 23) by ethanol

extraction of Leptospira biflexa suspensions. As demonstrated by Cox et al. (21) the hemolytic test utilizing this antigen is as sensitive an indicator of the presence of leptospiral antibodies as the agglutination-lysis test. Rothstein and Hiatt (56) postulated their "S" antigen (somatic) largely comprises the ESS of Chang and McComb (14) and therefore Cox's erythrocyte sensitizing antigen.

Attempts to discover the antigenic make up of leptospirae have been confined largely to chemical analysis of the organisms and interpretation of qualitative cross agglutinin absorption tests. The use of quantitative serological techniques in the study of leptospiral antigens and antibodies have never been reported.

The technique of quantitative chemical determination of agglutinin nitrogen was established by Heidelberger and Kabat (34, 35) who applied their method to the study of pneumococci. The same method of antibody estimation has been used in the study of brucellae (63), of Hemophilus influenza (2), of hemolytic streptococci (38), of gonococci (65), of meningococci (42), of Proteus (16), of Sh. dysenteriae (51), of S. typhosa (30) and of Staphylococcus aureus (43). There is reason to believe, then, that such a method can be used in the study of leptospirae.

MATERIALS AND METHODS

Preparation of Antigen: The leptospiral species used in this study, L. pomona, strain W, L. canicola and L. icterohaemorrhagiae AB, were isolated from single colonies on Cox's solid medium (22) and then carried in Stuart's broth medium (66) with weekly transfers. Approximately 5 ml of a 2 to 4 day old stock culture were used to inoculate 400 ml of Stuart's medium contained in a 1000 cc Owens Oval bottle. In 6 to 9 days these cultures were checked for contamination by dark field examination and by inoculation of thioglycollate broth. To pure cultures, as determined by microscopic examination, 0.2 per cent neutral formalin (3) was immediately added.

After standing for 48 hours at room temperature the cultures, which showed no growth in thioglycolate broth, were centrifuged at the same temperature in an International, horizontal type, size 2 centrifuge at 2500 rpm for 30 minutes. The supernatant fluids were decanted and centrifuged in the cold (1°C) in a Lourdes superspeed model AX angle Centrifuge at 10,000 rpm for 20 minutes. After decanting the supernatant fluids, the sedimented cells were resuspended in phosphate buffered saline (.15M, pH7.3) containing 0.025 per cent neutral formalin. The cells were then washed three times under the same conditions with buffered formalin saline. The leptospirae were finally suspended in buffered saline containing merthiolate (1:10,000) as preservative

and then stored at 1°C. Immediately before use the antigen was sedimented, resuspended in buffered saline and then lightly centrifuged to remove large "clumps" of organisms. The final cell suspension was a 50 to 90 fold concentration of the antigen.

Preparation of Antisera: Antisera against L. pomona (W), L. canicola and L. icterohaemorrhagiae AB were produced in rabbits using the following procedure. Consecutively, at five day intervals, 1 ml, 2 ml, 4 ml, and 6 ml of 5 to 7 day old culture of viable leptospirae were inoculated intravenously into rabbits. Five days following the last injection the rabbits were completely exsanguinated by cardiac puncture. Sera were filtered under atmospheric pressure through a Hercules filter disc type ST, and stored at 20°C. Just prior to use the sera were centrifuged for 30 minutes at 10,000 rpm and 1°C.

The ethanol fractionation technique of Deutsch (24) was used to separate the antibody active proteins from sera. The γ_2 serum globulin fraction was used in part of this study.

The microscopic tube agglutination test procedure was used for determining the titer of antisera and for testing the supernatant fluids of adsorbed sera for the presence of antibodies. The antigens consisted of formalized, washed cells, suspended in buffered saline containing 0.01 per cent merthiolate and having an optical density of approximately 0.01 as read on the Bausch and Lomb Spectronic 20 colorimeter

at 650 mμ. Sterile 0.85 per cent saline was used to make 10 fold serial dilutions of serum in a total volume of 0.1 ml using a single pipette for diluting, and to this was added an equal volume of antigen. The tubes were incubated over night (12 to 18 hours) at room temperature and their contents were examined microscopically at 100 x using a microscope fitted with an Abbe condenser into which was fitted a star diaphragm. Reactions of 25 per cent agglutination or more were considered in determinations of end point titer. The reciprocal of the serum dilutions, dilution of serum before the addition of antigen and not the final dilutions (2 x serum dilution), are recorded in the tables and figures. This procedure is referred to as the routine method.

In the special study of the agglutination test 4 and 2 fold serial dilutions in a total volume of 0.5 ml were used with a change of pipettes for each dilution and to this an equal volume of antigen was added. A non serial dilution method was also used (Table 7, column 5). It varied from the special method only in that dilutions were made by introducing directly into tubes containing varying amounts of diluent the exact amount of antisera required to make the desired dilution.

Protection test: L. pomona strain W was originally isolated by inoculation of the urine from an infected cow into guinea pigs (48) and then this strain was maintained in continuous guinea pig passage for approximately 3 years.

Sheep were inoculated with guinea pig blood containing these organisms and leptospirae were isolated from sheep blood, collected during the febrile stage of infection, by inoculation of Chang's fluid medium (15). After the fourth passage in media these organisms killed hamsters (5). Hamsters were inoculated with 1 ml of dilutions of antisera produced in rabbits against this L. pomona strain W variant. Some controls received 1 ml of normal rabbit serum, others received no serum, and all hamsters received 1 ml of the lethal organisms two hours following intraperitoneal inoculation of serum.

The quantitative test is essentially the same as that described by Kabat and Meyer (41). Duplicate samples were prepared in 7 ml Pyrex glass, round bottom centrifuged tubes as follows: to 1 ml of diluted serum were added 2 to 4 ml of the uniformly mixed leptospiral suspension. Two controls were set up; one contained an equal volume of the suspension plus saline in place of serum and the other consisted of serum plus saline without leptospirae. The tubes were closed with rubber serum vial stoppers and incubated for 2 hours in a 37°C water bath with mixing by inversion every 15 minutes. The tubes were refrigerated at 0°C for approximately 24 hours with occasional mixing, and then centrifuged at 10,000 rpm for 30 minutes in a PR 1 International centrifuge using the 6 place angle head in the multi speed attachment. The supernatant fluid was decanted, the tubes were

placed in an ice bath and the precipitate was resuspended in ice cold 0.9 per cent NaCl solution and allowed to stand for 30 minutes. The tubes were centrifuged again, the supernatant fluid decanted, and the precipitates washed in a similar manner twice more. The precipitates were then suspended in water and transferred quantitatively to micro-Kjeldahl flasks with the aid of distilled water containing a few drops of 1 normal sodium hydroxide. The micro-Kjeldahl analysis for nitrogen was carried out according to the method described by Heidelberger and Kabat (34, 35) except that the colorimetric (33) instead of the titrimetric method was used following steam distillation of the samples. The nitrogen value obtained was the combined nitrogen content of the antigen and antibody. Antibody nitrogen or agglutinin nitrogen is obtained by substracting the milligrams of antigen nitrogen added from the total nitrogen. Thus: milligrams agglutinin N = N determined − N in leptospiral suspension blank. Agglutinin N x 6.25 = agglutinin in mg protein.

With purified antibody solutions agglutinin nitrogen content was determined by measuring the nitrogen of the antibody solution before and following specific adsorption. The decrease in the nitrogen content of the supernatant fluid following agglutination and centrifugation was due to the carrying down of the antibodies specifically adsorbed to the antigen. The difference, then, between the nitrogen

of the unadsorbed antibody solution (control) and the adsorbed samples is a measure of the antibody nitrogen. The nitrogen content of the supernatant fluids was determined by the method of Lowry et al. (45) using Folin Ciocalteu Phenol Reagent. Dilutions of Armour's serum bovine albumin solution were used to make a standard curve which related color intensity produced by the phenol reagent, as measured on the Bausch and Lomb Spectronic 20, to milligrams nitrogen as determined by the micro-Kjeldahl analysis of identical samples.

Antigen nitrogen was determined by the method of Boyd for precipitates (11) using the phenol reagent. The mg N/ml of dilutions of washed antigens were determined by micro-Kjeldahl analysis and related to the optical density of the phenol reagent for these samples by a standard curve.

RESULTS

Before quantitative studies could be conducted certain preliminary tests and standards had to be made. A standard curve relating the optical density of dilutions of the phenol reagent serum albumin solution to micro-Kjeldahl N was constructed. Also a curve was made which related the optical density of phenol reagent leptospiral cell suspensions to micro-Kjeldahl N. A study was made of the routine agglutination test and the conditions of time, temperature and optimal proportions necessary for complete adsorption of antisera. Cross agglutinin adsorption tests were conducted to ascertain qualitative relationships. The quantitative tests were used to estimate the amount of homologous and heterologous antibody present in a given antiserum and for the study of the combining ratio of leptospiral N to antibody N.

In order to obtain easily and quickly an estimate of the milligrams of nitrogen per ml of an antigen, a graph was made which relates the optical density of various dilutions of a cell suspension with its micro-Kjeldahl nitrogen (Table 1 and Figure 1). Although formalized, washed L. canicola suspensions were used for constructing the curve, many other determinations were made with other serotypes and these values correlated well with the established curve.

Figure 2 is the standard plot used to convert optical density of the protein phenol reagent solution to milligrams of nitrogen. Both the spectrophotometric (68) and the

micro-Kjeldahl methods were used to determine the total nitrogen of identical two fold diluted samples of the serum albumin solution. The spectrophotometric method measures milligrams protein, therefore milligrams nitrogen was calculated by multiplying the measured value by 0.16. Two separate determinations were made (Table 2), but corresponding values were averaged and used to plot the graph (Figure 2).

The color intensity of leptospiral cell phenol reagent suspensions were plotted against the micro-Kjeldahl nitrogen content of identical suspensions (Table 3 and Figure 3). Formalized L. icterohaemorrhagiae AB, washed cells, suspended in sterile 0.9 per cent saline were used for the determination. The optical density readings and the mg N/ml listed in Table 3 compare well with those recorded in Table 1 for a different antigen.

Quantitative estimations of the antibody nitrogen of antisera were made by two separate methods. With whole serum, the amount of N in the antigen and the specific precipitate was determined by direct micro-Kjeldahl analysis. But with purified antibody solutions, the amount of nitrogen in the supernatant fluid before and following adsorption was measured by the phenol reagent method and the difference in nitrogen was converted by an albumin standard to micro-Kjeldahl nitrogen. In the latter procedure the nitrogen of the antigen was obtained by converting optical density of the cell phenol reagent solution, by a standard curve

(Figure 3), into micro-Kjeldahl nitrogen. Since part of the determinations of antibody nitrogen were made by measuring the color intensity of the phenol reagent related to the micro-Kjeldahl nitrogen of serum albumin, serum globulin and leptospiral cells, it is necessary, if the results of both methods are to be compared, to ascertain the relation ship between these components regarding the ratio of phenolic groups to total nitrogen. If the ratio of phenolic groups to total nitrogen is the same in serum albumin as in suspensions of leptospirae, then the values of a and a' in the following equations should be approximately the same.

$$\frac{PRA}{MKA} = a \qquad \frac{PRL}{MKL} = a'$$

PRA - optical density of albumin phenol reagent solution

MKA - micro-Kjeldahl nitrogen of albumin solution

L - leptospirae suspension

Several values of a and a' derived from samples selected from Figures 2 and 3 respectively are listed in Table 4. The difference in the two ratios is approximately 0.5, which means a variance of 0.01 milligram of nitrogen. This value is beyond the accuracy of the micro-Kjeldahl method, therefore the two ratios can be considered to be equal. This was not the case when a comparison was made of the ratios (phenolic groups to total nitrogen) observed with serum albumin and purified antibody solutions (actually serum globulin) (Table 5). The values in Table 5 were chosen

arbitrarily from Figures 2 and 4. The difference in ratios a and a' is approximately 5.4 which represents about 1 milligram of nitrogen. Therefore, antibody estimations by micro-Kjeldahl analysis of specific precipitates cannot be compared with antibody determinations by the phenol reagent method using serum albumin as a standard. Consequently, the method used to measure antibody nitrogen will be given in each case.

It was determined that three washings were necessary to remove the extraneous nitrogen containing compounds of the media from the leptospiral cells. The results of micro-Kjeldahl analysis of 3 ml samples from successive washings of cell suspensions are shown in Table 6. Also three washings of specific precipitates were found to be adequate to remove the serum components not specifically adsorbed to the antigen (Table 6).

An attempt was made to relate the agglutination titer of antisera, using 10 fold serial dilutions (routine method), with its milligrams of antibody nitrogen. The results of 14 determinations using micro-Kjeldahl analysis of specific precipitates are plotted in Figure 5. The graph shows very little relation between milligrams of antibody nitrogen and agglutination titer. A line can be drawn only if zero titer is assumed to represent zero antibody nitrogen and zero is used as a point of departure.

Since the method of nitrogen analysis is known to be accurate, a study of the agglutination test was made. The

titer of an antiserum was shown to vary significantly depending on both the method of diluting the serum and the type of antigen used (Table 7). Using the routine method of making ten fold serial dilutions of antisera, titers which were 100 times higher were observed with live antigens as compared to formalized suspensions (columns 1 and 2). The ten fold serial dilution (routine) method resulted in considerably higher titers than 4 fold and 2 fold serial dilutions. On the average the 10 fold dilution method gave titers approximately 10 times higher than the 4 fold method and 100 times higher than the 2 fold method (columns 2, 3 and 4). Titers observed by the 4 fold dilution method were about 5 times higher than those obtained with 2 fold dilutions (columns 3 and 4). In column 5 serial dilutions were not used, instead dilutions were made by introducing directly into tubes containing varying amounts of diluent the exact amount of antisera required to make the desired dilution. There was no significant difference in titer between the two methods of making 4 fold dilutions of antisera (columns 3 and 5).

Retesting some of the samples plotted in Figure 5 using the 2 fold serial dilution method resulted in a better agreement between agglutination titer and milligrams antibody nitrogen (Figure 6).

The constant antibody optimal proportions method, a modification (10) of the method of Dean and Webb (23) was

used for each antiserum studied to estimate the antigen antibody ratio which would result in complete adsorption. After the optimal proportions point was determined, incubation in a water bath at 37°C was continued for 2 hours and then the tubes were placed in the refrigerator over night. Following centrifugation (2500 rpm for 15 minutes) the supernatant fluids were tested for the presence of agglutinins. Typical results are recorded in Table 8. The most rapid agglutination occurred with a 1:2 dilution of antigen and a 1:50 dilution of antibody solution (tube 2). The optimum ratio of antigen to serum dilution was 0.5/0.25 x 2/50 = 0.08. Or, the optimal proportion may be expressed as 1/2/1/50 = 50/2 or 1/0.04. This is the method used to express the optimum ratio for precipitin reactions. For agglutination reactions, however, it would be better to express the ratio of antiserum to antigen. In this case the ratio is 1/25; one unit of serum reacts optimally with 25 units of the original antigen when they are mixed in equal amounts. Note in Table 8 that complete adsorption occurred not only in tube 2, but also in tubes 3, 4 and 5. Therefore, there is a range of antibody to antigen ratios usable in adsorption procedures extending, in this case, from 1/25 to 1/10. Excess antigen has a marked effect on the adsorption process. Tubes 1 and 2 differed in agglutination time by only 25 hundredths of a minute and differed in antigen concentration by 2 fold. But complete adsorption occurred in tube 2 and

not in tube 1. Tubes 4 and 5 differed from tube 2 in time by 2 or more minutes and in antigen concentration by 2 or more fold, but complete adsorption occurred in all three tubes.

To determine the antibody nitrogen of a purified antibody solution, a 1:10 to 1:20 dilution of serum globulin was required. To apply the optimal ratio to this requirement, 2 ml of 1:16 dilution of antibody was added to 2 ml of the undiluted antigen.

The qualitative antigenic relationships among the three serotypes, L. pomona, L. icterohaemorrhagiae AB, and L. canicola, were established by cross agglutinin adsorption tests (Table 9). In each case the antigens removed all of the cross reacting antibodies from the homologous antisera, but the heterologous antigens did not remove all of the homologous antibodies. The heterologous antigens did reduce the homologous reaction and the other heterologous titer in every case. With one exception, a heterologous antigen did not exhaust a serum of the antibodies reactive with the second heterologous antigen. The exception was: L. icterohaemorrhagiae AB antigen removed all the antibodies from L. pomona antiserum that were reactive with L. canicola.

Quantitative estimations of the milligrams of agglutinin N present in several antileptospiral (rabbit) sera which reacted with the homologous and heterologous antigens are shown in Table 10. In general the results indicate that

L. pomona is more similar antigenically to L. icterohaemorrhagiae AB than to L. canicola. The latter two species, however, are more closely related antigenically than L. pomona and either of the other two serotypes. The extent of cross reaction indicates a constant reciprocal antigenic relationship among the three serotypes. For example, L. icterohaemorrhagiae AB antigen removed approximately the same amount of agglutinin N from L. pomona antiserum as L. pomona antigen removed from L. icterohaemorrhagiae AB antiserum. From these data and the results of the cross adsorption tests, a probable antigenic scheme of these serotypes can be proposed (vid. discussion).

Some information concerning the combining ratios of leptospirae and their homologous antibodies was sought using quantitative methods. Increasing amounts of a suspension of L. icterohaemorrhagiae AB of known N content were added to a series of tubes each containing a given volume of homologous antibody solution. The quantity of antibody N precipitated by each amount of antigen was determined by the phenol reagent method (Table 11). As the amount of antigen added was increased the amount of antibody N precipitated increased correspondingly. Proportionately more antibody was removed by low concentrations of antigen than with heavier suspensions. When milligrams of antigen nitrogen added were plotted against the milligrams of antibody nitrogen precipitated a curve was obtained (Figure 7). The arrows show

the points at which complete adsorption occurred. When the ratio of milligrams antibody nitrogen to milligrams antigen N was plotted against the corresponding milligrams of antigen added a straight line relationship was obtained (Figure 8). An empirical equation was derived by Kabat and Meyer (41) which expressed this relationship. By means of this formula the milligrams of antibody nitrogen precipitable by each amount of antigen added can be calculated for the antibody excess zone.

Antibody N precipitated = $ax \quad bx^2$

x = the amount of antigen N added

Refer to Figure 8:

a = intercept on the Y axis 1.56

b = slope of the line 4.8

When $x = 0.022$ then,

AbN precipitated = $(1.56)(0.022) = (4.8)(0.022^2)$

$= 0.033 - 0.002 = 0.031$

The values listed in column 5, Table 11, were obtained in this manner. There is 4 to 20 per cent variation between experimental and calculated values. Since this empirical equation is based on the slope and Y axis intercept of the straight line, then the degree of agreement between experimental and calculated values would depend on how well the straight line represented the plotted points. It can be observed from Figure 8 that the arrangement of the points do not conform well to a straight line, therefore variation

was to be expected between values calculated from the empirical formula and the experimental results. Better correlation between calculated and experimental values was observed when the theoretical equation derived by Heidelberger and Kendall (37) was applied to these data (vid. discussion and Table 11, column 7).

The power of an anti-L. pomona (W) rabbit serum to protect hamsters against the homologous organisms was compared to its milligrams of antibody nitrogen (Table 12). One ml of 1:1000 dilution but not a 1:5000 dilution of the antiserum having an original titer of 10^{-5} protected hamsters against 1 ml of the lethal suspensions. A 1:1000 dilution of antiserum contained 0.005 milligrams antibody nitrogen per ml.

DISCUSSION

Studies of the qualitative and quantitative aspects of the leptospiral antigen antibody system revealed the usual characteristics found in other bacterial antigen antibody systems. Some variations from the typical reaction were observed and these differences are pointed out. The observations reported here, however, are in accord with the findings of others working with leptospirae and in some cases this work supports or amplifies their results.

Quantitative studies of leptospiral antisera revealed little relations between the agglutinin titer using the routine method and the quantity of antibody nitrogen present. Heidelberger et al. (34) found in an heterologous system that the ratio of agglutinin titer to total antibody N of a type I antipneumococcal serum did not compare with the ratio of agglutinin titer to antibody N of a type I R (rough) anti serum. They explained that these variations in ratios with different organisms are probably a manifestation of differences in the combining ratios of the respective antigen antibody systems and of the amount of each antigen at the bacterial surface.

With an homologous system, however, Barrett and Tripp (4) found a relation between the agglutinin titer using 2 fold serial dilutions and the total agglutinin nitrogen content of several type I antipneumococcal sera. Approximately the same degree of relation was observed by others working

with homologous antigen antibody systems of S. typhosa (30) and Staphylococcus aureus (43). In our work, when 2 fold serial dilutions were made by using a clean pipette for each dilution, the ratio was comparable to that observed by the above workers. From the fact that the agglutination test is considered accurate only within 1 tube dilution of the end point (41) and in consideration of the variation in titers obtained by different dilution methods, it is apparent that our routine method of serologic testing resulted in considerable error in the estimation of antibody content.

Generally it is stated that formalin does not noticeably affect the antigenicity of most substances (11). But the obvious difference between tests of sera using live and formalized antigen must be attributed to the action of formalin on the properties of the leptospirae. Formaldehyde reacts with the amino group of amino acids and probably with those of proteins (32). The probable structural equation representing this combination follows:

$$R\text{-}NH_3^+ + HCHO \longrightarrow R\text{-}NH_2\text{-}CH_2OH$$

While the formaldehyde molecules may react with the combining sites of the cell and thereby prohibit antibody attachment, there is no evidence for this. The formalin may merely block nonspecifically a point of attachment. Combination of formaldehyde and amino groups can also occur in this manner:

$$R\text{-}\overset{H}{\underset{H}{N}}\text{-}CH_2\text{-}\overset{H}{\underset{H}{N}}\text{-}R'$$

and thus block or modify an antigen site.

Microscopic examination revealed marked differences in the type of agglutination. The live antigens were tightly bound into large aggregated whereas formalized cells were loosely agglutinated into small groups. Agglutination of heat killed cells (50°C for 10 minutes) was similar to that of formalized cells and tests of sera with these altered antigens gave comparable results. It does not necessarily follow that formaldehyde and heat produce the same changes in the leptospirae, but Jacobs and Sommers (40) observed that the serologic changes due to formalin resembled the alterations due to heat.

Consistent results were not obtained from quantitative analysis of the specific precipitates of antibody from whole serum and heterologous antigens. Considerable difficulty was encountered in recovering the total agglutinated mass for analysis, since small particles were continually separating from the main mass during washing and decanting procedures. Similar difficulties were reported by others working with the Neisseria group (42) and with pneumococci (34). We found that nitrogen determinations of paired samples of heterologous reactants varied from 20 to 30 per cent and therefore could not be considered reliable. The precipitates formed by homologous reactants were more stable and adhered more firmly to the glass tube. The analysis of paired samples of homologous systems were consistent.

In order to determine more accurately the amount of antibody reactive with heterologous antigens, therefore, it was necessary to use purified antibody solutions and measure the nitrogen of the supernatant fluid before and following specific adsorption. The difference between the two values was calculated as the amount of antibody nitrogen precipitated.

Three methods of preparing antibody solutions were tried. The Felton (26) and the salt precipitation methods resulted in either too great a loss of antibody or such a concentration of protein that the phenol reagent method of analysis could not be used. The aqueous ethanol fractionation technique of Deutsch (24) proved suitable for the separation of immune γ_2 globulin fraction from rabbit sera. Since leptospiral antibodies are found in both the γ_1 and γ_2 fractions (6), some loss of antibody activity was expected and generally amounted to a 5 fold decrease in titer.

It was observed that a range of optimal ratios of antibody to antigen exists in which complete adsorption of agglutinins occurred. Miles (46) interprets such a phenomenon as an indication of a multiplicity of antigen antibody systems. From this and reports on the antigenic analysis of leptospirae (61, 56, 9, 57, 29, 20), it can be concluded that these organisms contain more than one antigenic component and probably contain many antigenic factors.

As an increasing amount of antigen was added to a constant amount of the antiserum a zone of complete precipita-

tion of agglutinin was reached and further addition of antigen resulted in incomplete adsorption of the serum. In agglutinin adsorption methods the supernatant fluid cannot be adequately examined for excess antigen because centrifugation of the specific precipitate may result in the sedimentation of unagglutinated organisms. It is difficult to know, then, whether the zone of complete precipitation of antibody (determinations 7 and 8) is the equivalence zone or where the equivalence point may be located. The curve relating antigen added to antibody precipitated does not indicate clearly an equivalence area or point which would be identified by a "flattening" of the curve to the horizontal near maximum antibody precipitation. These findings could be explained by the existence of several antigen-antibody systems having different equivalence points.

For the purpose of applying the theoretical formulation of Heidelberger and Kendall (37) to these data, it will be assumed that the point of maximum precipitation of antibody (determination #8) is the equivalence point. The following is an expression based on the mass law of chemical reactions derived by Heidelberger and Kendall (37) and reported to describe the agglutination reaction in the region of excess antibody (41).

$$AbN = 2RX \quad \frac{R^2}{A} X^2$$

AbN = antibody nitrogen precipitated

X = antigen precipitated, in terms of nitrogen for

protein

R = ratio $\frac{\text{antibody N}}{\text{antigen N}}$ at the equivalence point

A = total antibody N in unit volume of serum

The factors "a" and "b" from the empirical formula (vid. Results) are related to the above equation:

$$a = 2R$$

$$b = \frac{R^2}{A}$$

Therefore, the amount of antibody N precipitable by a given amount of antigen can be calculated when the total antibody N content of the serum and the ratio of antibody N to antigen N at the equivalence point are known for a given antigen-antibody system. The calculated and determined values differ by no more than 0.01 mg N per ml or approximately 10 per cent. The calculated values are considered accurate only in the region in which a change of ratio was observed (41); thus, the calculated values of antibody precipitated in samples 7 and 8 were not in agreement with experimental results.

The experimental value for "a" was greater than the value for "2R", but according to Heidelberger and Kabat (36) the "2R" value should be approximately 1.2 times "a". These workers found approximately 2 per cent difference between experimental and calculated values, but in our work 4 to 12 per cent variation was observed and the calculated value for total agglutinin nitrogen differed from the experimental

results by 60 per cent. It is apparent, then, that the leptospiral antigen antibody system does not conform well to the theoretical formulation for homogenous systems. Stokinger et al. (65) working with Neisseria gonorrheae found such a lack of correlation between the quantitative agglutinative data and the values calculated from the mathematical theory. They observed that a linear relationship was not always obtained and their calculated values for total antibody nitrogen varied from 2 to 40 per cent of that found by experiment.

The results of cross adsorption tests indicated either that the three serotypes have identical antigen components or chemically related antigens present. Assuming that each contains one or more common antigens, it is possible to propose antigenic structures for the three serotypes which comply with the relationships observed.

L. icterohaemorrhagiae AB	a b c . . .
L. canicola	a c d . . .
L. pomona	b c e . . .

These formulae are the minimum antigenic compositions which explain the cross reactions, and certainly more specific and/or cross reacting antigens or antigenic components may be present in any of these serotypes. Note, however, in this scheme that the three serotypes contain antigen c in common and that L. canicola and L. pomona each have in common with L. icterohaemorrhagiae AB one other antigen,

i.e. antigen a and b respectively. Therefore, when L. pomona antiserum was adsorbed with L. icterohaemorrhagiae AB antigen, the antibodies reactive with antigen c were removed and L. canicola cells were not agglutinated by the adsorbed sera. The reverse was also true. L. canicola antiserum adsorbed with L. icterohaemorrhagiae AB cells failed to agglutinate L. pomona antigens.

The data on the per cent of cross reaction among the species supports the hypothesis of these antigenic formulae. It is also observed from these data that antigen a is a major antigen in L. icterohaemorrhagiae AB and L. canicola and that antigen e constitutes the major antigenic component(s) of L. pomona. It would follow also that antigen components b, c and d are minor fractions in their respective serotypes. But Miles (46) cautions that interpretation of quantitative data in this manner assumes that there is a quantitative correspondence between the different kinds of antibodies in a serum and the different kinds of antigens present in the homologous bacterium. He pointed out, the antibody response to many repeated doses of a small amount of antigen may be as great as that to many doses of a large amount, so that after prolonged immunization with a bacterium containing a major and minor antigen the corresponding antibodies may be in equal concentration in the serum.

Gispen and Schüffner (29) proposed antigenic factors A and B for L. icterohaemorrhagiae AB and Savino and

Rennella (57) considered that it contains a specific antigen b and two genus or serogroup specific antigens z and x which are shared with certain other leptospirae. L. icterohaemorrhagiae A the incomplete biotype of L. icterohaemorrhagiae AB (9) cross reacts with L. canicola to even a greater extent than the latter does with the complete biotype (71). Considering all the available data it could be proposed that Gispen's A antigen and our antigen a may be Savino's z and x antigens, and Gispen's B factor and our antigen b may be synonomous with Savino's b factor. Thus, L. icterohaemorrhagiae A would contain factors z and x and since L. canicola is more closely related to it than to L. icterohaemorrhagiae AB it probably contains more antigen x or z than the latter but not both. In summary, the possible antigenic structures based on our results and other reports may be as follows:

L. icterohaemorrhagiae AB	a (x, y), b, c . . .
L. icterohaemorrhagiae A	a (x, y), b
L. canicola	a (x), c, d . . . .
L. pomona	b, c, e . . . . .

These formulae were constructed on the assumption that cross reactions were solely due to common (identical) antigens present in each serotype. It is equally as possible that the heterologous reactions are due in part to chemically similar antigens. As Kabat and Meyer stated (41) only in the latter case are true cross reactions involved.

In summation, the qualitative examination of this antigen-antibody system indicated the presence of multiple antigens in the leptospiral cell, and the relationship of the serotypes studied. The usual quantitative serological method of measuring antibody content of whole serum can be used only with homologous reactants. For the quantitative study of heterologous reactions, the phenol reagent method of analysis using purified antibody solutions is required. The quantitative study supported the qualitative results and aided in the interpretation of them. Also, it pointed out variations of the leptospiral antibody antigen system from the typical results of other bacteria which have been studied.

SUMMARY AND CONCLUSION

Qualitative and quantitative examination of L. icterohaemorrhagiae AB, L. canicola and L. pomona were conducted to ascertain their antigenic interrelationships. Before quantitative studies of the leptospiral antigen antibody system could be conducted certain preliminary tests and standards had to be made. Three standard straight line graphs were constructed. One related optical density of antigen suspensions to their total nitrogen content. Also the optical density of various leptospiral suspensions dissolved in phenol reagent solutions were plotted against their total nitrogen, expressed in milligrams. Another plot was made which related the optical density of dilutions of albumin phenol reagent solution to milligrams nitrogen. A study of the agglutination test showed that the routine procedure produced significant error in the estimation of antibody. The conditions of time, temperature and optimal proportions necessary for complete adsorption of antisera were determined. A method was devised for the preparation of antigens which were suitable for quantitative procedures. Reciprocal cross agglutinin adsorption tests indicated qualitative interrelationships among the three serotypes and furnished sufficient information for the construction of probable antigenic structures.

The usual quantitative method of estimating the anti-

body content of whole serum could be used only for the homologous system. A different procedure using purified antibody solutions was required to measure the degree of cross reaction. The homologous antibody content of a serum and the amount of this antibody which combines with the two heterologous serotypes were quantitatively measured. A quantitative study of the combining ratio of antigen N to antibody N was conducted. These data aided in the interpretation of qualitative results.

Formaldehyde caused some change in the leptospiral cell which can be observed by serologic examination. Since formalin affects antigen-antibody combination, relationships derived from these experiments cannot be applied to the organisms in their natural state without qualification. It can be concluded from these data, however, that the leptospiral cells contain more than one antigenic component and probably contain several antigens. Part of the cross reactions of these serotypes may be explained by chemically related antigens. L. icterohaemorrhagiae AB and L. canicola are more closely related antigenically than L. pomona and either of the former serotypes.

TABLE 1

COMPARISON OF THE OPTICAL DENSITY[1] OF LEPTOSPIRAL SUSPENSIONS[2] AND THEIR MICRO KJELDAHL NITROGEN CONTENT

Optical Density	mg N/ml	Optical Density	mg N/ml
0.92	0.095	0.23	0.026
0.75	0.081	0.17	0.023
0.54	0.054	0.113	0.016
0.48	0.052	0.105	0.009
0.34	0.041	0.045	0.006
0.23	0.017		

1. Optical density was determined on a Bausch and Lomb Spectronic 20 colorimeter at 650 mμ.

2. Formalized L. canicola, washed three times in buffered saline containing 0.025 per cent formalin, suspended in 0.9 per cent NaCl solution.

TABLE 2

COMPARISON OF COLOR INTENSITY OF ALBUMIN PHENOL REAGENT SOLUTIONS WITH TOTAL NITROGEN

Albumin[1] Dilution	Optical Density[2] Phenol Reagent #1[3]	#2	Spectrophotometric Method #1	#2	Micro-Kjeldahl Method #1	#2
1:40			0.271	0.254	0.234	0.230
1:80	0.71	0.725	0.134	0.128	0.108	0.117
1:160	0.385	0.390	0.075	0.067	0.066	
1:320	0.213	0.222	0.034	0.034		
1:640	0.100	0.110	0.017	0.019		
1:128	0.050	0.067	0.009			

1. Armour's 5 per cent bovine serum albumin solution.
2. Bausch and Lomb Spectronic 20 colorimeter at 660mμ.
3. Determinations #1 and #2 were averaged and used to make Figure 2.

TABLE 3

OPTICAL DENSITY[1] OF LEPTOSPIRAL CELL PHENOL REAGENT SUSPENSION[2] RELATED TO MICRO-KJELDAHL NITROGEN

Cell Phenol Reagent Optical Density	Micro-Kjeldahl mg N/ml	Optical Density of Cell Suspension
0.94	0.186	1.4+
0.59	0.095	0.92
0.55	0.081	0.75
0.36	0.054	0.54
0.28	0.047	0.44
0.24	0.038	0.36
0.18	0.028	0.26
0.12	0.018	0.15
0.05	0.006	0.05

1. Bausch and Lomb Spectronic 20 at 660

2. L. icterohaemorrhagiae AB formalized, washed and suspended in 0.9 per cent saline.

TABLE 4

COMPARISON OF THE RATIO OF PHENOLIC GROUPS TO TOTAL NITROGEN IN SERUM ALBUMIN[1] AND LEPTOSPIRAL CELLS[2]

Determination #	Optical Density A[3]	Optical Density L[4]	Total Nitrogen A	Total Nitrogen L	Ratios[5] a	Ratios[5] a'
1	0.8	0.82	0.135	0.128	5.91	6.40
2	0.68	0.64	0.115	0.100	5.91	6.40
3	0.59	0.55	0.100	0.086	5.90	6.39
4	0.43	0.41	0.73	0.064	5.90	6.40
5	0.22	0.18	0.03[8]	0.028	5.80	6.44
Column	1	2	3	4	5	6

1. Armour's 5 per cent bovine serum albumin solution.
2. Formalized, washed leptospirae suspended in saline.
3. A - albumin; values obtained from Figure 2.
4. L - leptospirae; values obtained from Figure 3.
5. $a = \frac{\text{values in column 1}}{\text{values in column 3}}$ $a' = \frac{\text{values in column 2}}{\text{values in column 4}}$

TABLE 5

COMPARISON OF THE RATIO OF PHENOLIC GROUPS TO TOTAL NITROGEN IN SERUM ALBUMIN[1] AND ANTIBODY SOLUTION[2]

Sample #	Optical Density[3] A[5]	G[6]	Total Nitrogen[4] A	G	Ratio a	a'
1	0.8	0.65	0.135	1.36	5.91	0.47
2	0.68	0.46	0.115	1.00	5.91	0.46
3	0.59	0.30	0.100	0.69	5.90	0.43
4	0.43	0.20	0.073	0.50	5.90	0.40
5	0.22	0.09	0.038	0.29	5.90	0.31
Column	1	2	3	4	5	6

1. Armour's 5 per cent bovine serum albumin solution.
2. Gamma globulin fraction of L. pomona antisera (rabbit)
3. Optical density of albumin phenol reagent solutions as read on a Bausch and Lomb Spectronic 20 colorimeter at 660 mμ.
4. Micro-Kjeldahl analysis
5. A - albumin; values obtained from Figure 2
6. G - globulin; values obtained from Figure 4
7. $a = \frac{\text{values of column 1}}{\text{values of column 3}}$ $a' = \frac{\text{values of column 2}}{\text{values of column 4}}$

TABLE 6

MILLIGRAMS OF NITROGEN REMOVED BY WASHING PROCEDURE

Wash #	Milligrams Micro-Kjeldahl Nitrogen[1]	
	Antigen Wash[2]	Supernatant Fluid[3] of Specific Precipitate
1	0.095	0.35
2	0.021	0.08
3	0	0
4	0	0
5	0	0

1. Five determinations were made and averaged to obtain these values.

2. Analysis of 3 ml of wash fluid.

3. Analysis of 1 ml of supernatant fluid.

TABLE 7

COMPARISON OF METHODS OF DILUTION WITH TITER OF LEPTOSPIRAL ANTISERA[1]

Serum #	Serial Dilutions[3] 10 fold		4 fold	2 fold	Non Serial[4] 4 fold
1	4[2]	4	3	2	3
2	5	4	3	2	3
3	5	5	3	3	3
4	6	5	3	3	3
5	7	4	3	3	3
6	7	4	3	3	3
7	6	4	3	2	3
8	6	5	3	2	3
9	7	4	3	2	3
10	7	5	3	2	3
11	6	4	3	2	3
Average	6	4.5	3	2.4	3
Column	1	2	3	4	5

1. L. pomona W antisera (bovine)

2. Live cultures of L. pomona W were used as antigen. Formalized, washed cells were used as antigen for the remaining tests. The numbers represent the negative exponent to the log base 10 of the serum dilution.

3. 10 fold serial dilutions were made using a single pipette. For 2 and 4-fold dilutions a different pipette was used for each dilution.

4. Dilutions were made by introducing directly into tubes containing varying amounts of diluent the exact amount of antisera required to make the desired dilution.

TABLE 8

OPTIMUM PROPORTIONS DETERMINATION

Tube #	1	2	3	4	5	6	7
Antigen[1]	0.5	0.25	0.17	0.12	0.10	0.05	0.05
Saline	–	0.25	0.33	0.38	0.40	0.45	2.45[5]
Antigen dilution[2]	1:1	1:2	1:3	1:4	1:5	1:10	1:50
Serum[3]	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Time of Agglutination (min.)[4]	2.0	1.75	3	4	5	10	–
Test for Agglutinins	+	–	–	–	–	+	+

1. Optical density approximately 1.2; mg N/ml = 0.126. Formalized L. canicola washed suspension approximately 50 x concentrated.

2. Exact or close approximate dilution

3. Rabbit anti-L. canicola purified antibody solution, diluted 1:50.

4. Agglutination determined by the unaided eye.

5. Only 0.5 ml of master dilution was transferred.

TABLE 9

CROSS AGGLUTININ ADSORPTION RESULTS WITH THREE ANTI-LEPTOSPIRAL RABBIT SERA[1]

Antiserum	Adsorbing Serotype	Titers[2] with Antigens L. pomona	L. icterohae-morrhagiae AB	L. canicola
L. pomona	none	8192	2048	1024
	L. pomona	0	0	0
	L. icterohae-morrhagiae AB	1024	0	0
	L. canicola	1024	128	0
L. icterohae-morrhagiae AB	none	32	1024	256
	L. pomona	0	512	128
	L. icterohae-morrhagiae AB	0	0	0
	L. canicola	4	128	0
L. canicola	none	32	1024	4096
	L. pomona	0	512	1024
	L. icterohae-morrhagiae AB	4	0	512
	L. canicola	0	0	0

1. Rabbits were inoculated with whole cultures of leptospirae.

2. Titers are expressed as the reciprocal of the highest serum dilution showing agglutination. "0" indicates complete adsorption as demonstrated by no evidence of agglutination at 1:20 serum dilution.

TABLE 10

QUANTITATIVE ESTIMATIONS
OF AGGLUTININ NITROGEN IN ANTI-LEPTOSPIRAL
SERA (RABBIT) FOR HOMOLOGOUS AND HETEROLOGOUS SEROTYPES

Antisera[1] for serotype	MG Antibody N Precipitated Per ML by Serotype Antigen		
	L. pomona	L. icterohae-morrhagiae AB	L. canicola
L. pomona	0.55[2]	0.14	0.08
per cent of homologous	–	25	14
L. icterohae morrhagiae AB	0.12	0.52	0.38
per cent of homologous	23	–	73
L. canicola	0.05	0.33	0.42
per cent of homologous	12	78	–

1. The γ_2 globulin fractions were used in adsorption tests.

2. Represents loss of nitrogen from the globulin solution due to precipitation of antibody with the adsorbing antigen. Corrected for serum blank and nitrogen present in antigen supernatant fluid. Paired samples were used and then averaged.

TABLE 11

AGGLUTININ N REMOVED BY INCREASING AMOUNTS OF L. ICTEROHAEMORRHAGIAE AB SUSPENSIONS FROM HOMOLOGOUS ANTIBODY SOLUTION

Determination	Antigen (Ag) N[1] added	N/ml of adsorbed solution	Antibody(Ab)N precipitated/ml[2]	Ratio AbN/AgN	Calculated AbN/ml	Test for excess antibody	Theoretical calculated AbN/ml
1	0.022	0.935[3]	0.037	1.70	0.031	+[4]	0.03
2	0.052	0.900	0.072	1.40	0.068	+	0.063
3	0.077	0.884	0.088	1.10	0.092	+	0.084
4	0.105	0.877	0.045	0.90	0.106	+	0.102
5	0.121	0.874	0.098	0.81	0.119	+	0.109
6	0.154	0.866	0.106	0.69	0.126	−	0.118
7	0.234	0.857	0.115	0.64	0.103	−	0.100
8	0.254	0.854	0.118	0.70	0.086	+	−
9	0.350	0.862	0.110	0.314	−		−
Column	1	2	3	4	5	6	7

1. Antigen nitrogen was measured by the phenol reagent method (Figure 3).

2. Difference between N content of adsorbed and unadsorbed antibody solution, minus the nitrogen content of the supernatant fluid of the antigen blank.

3. Corrected for reagent blank nitrogen and antigen control nitrogen.

4. (+) - antibody was present in supernatant fluid of adsorbed sera.
   (−) - complete adsorption of antibody.

TABLE 12

THE PROTECTIVE POTENCY OF L. POMONA ANTISERA (RABBIT) IN HAMSTERS AGAINST HOMOLOGOUS LETHAL ORGANISMS[1]

	Controls		Test Samples Immune Serum									
	No Serum	Normal Serum										
Serum dilution	–	1:10	Not diluted	1:4	1:16	1:64	1:256	1:512	1:1T[2]	1:5T	1:10T	1:15T
Titer	–	–	10^5	10^5	10^5	10^4	10^3	10^2	10^1	10^1	10^1	10^1
No. of animals	4	6	4	1	1	1	1	1	1	1	1	1
Death in days	5	5 to 14	–	–	–	–	–	–	–	5	5	5
MG Ab N/ml			1.15	–	–	–	–	–	0.005	–	–	–

1. 1 ml of a 4 day old culture was inoculated 2 hours following serum injections.

2. T = thousand

FIGURE 1. The Relation Between the Optical Density of Lepto spiral Cell Suspensions and their Micro Kjeldahl Nitrogen.

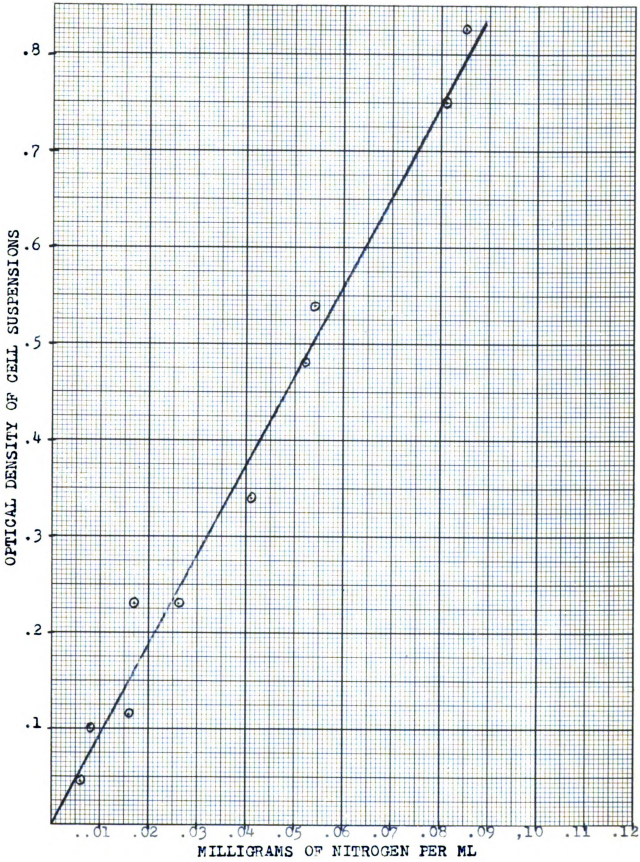


FIGURE 2. Optical Density of Serum Albumin Phenol Reagent Solutions Related to Total Nitrogen

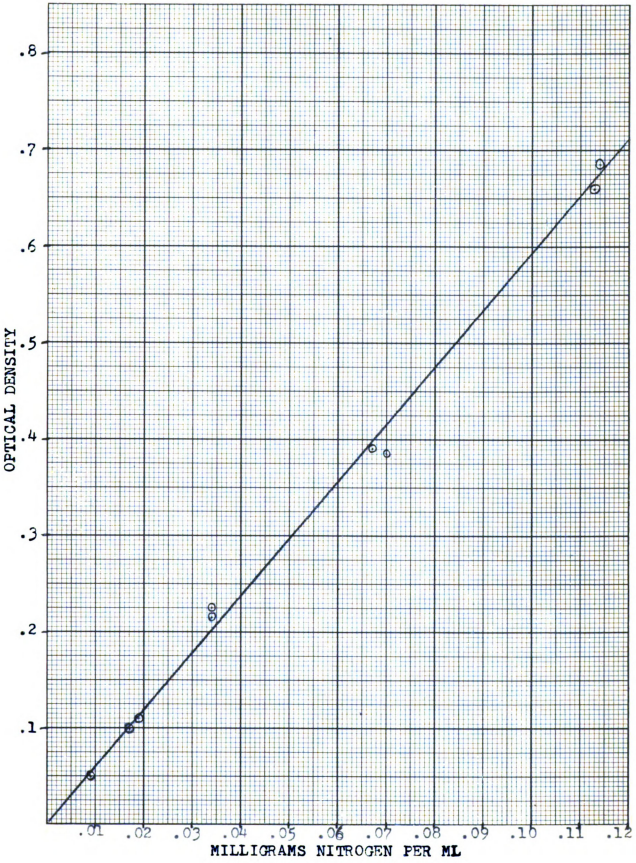


FIGURE 3. Optical Density of Leptospiral Cell Phenol Reagent Suspensions Compared to Total Nitrogen

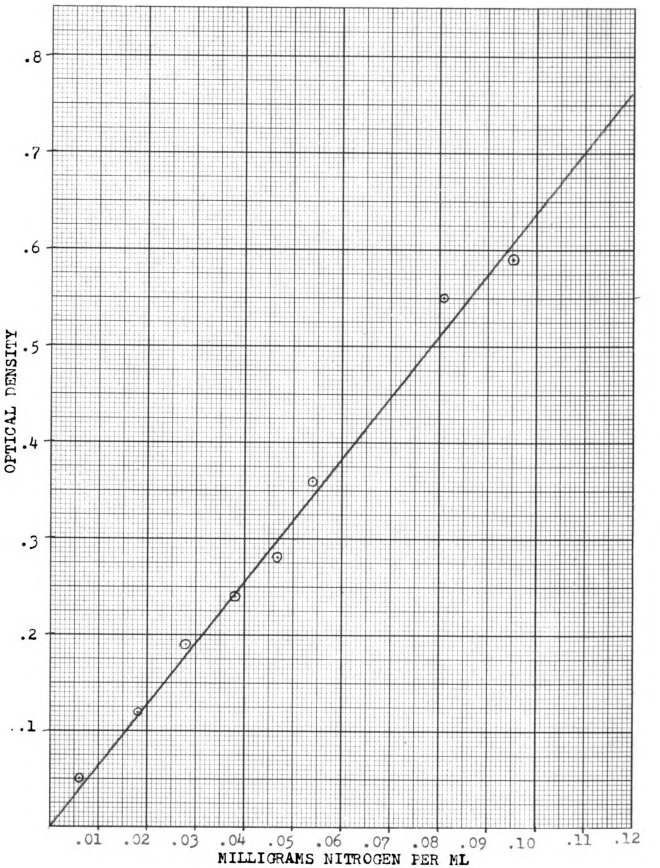


FIGURE 4. A Comparison of the Optical Density of Serum Globulin Phenol Reagent Solutions with their Micro-Kjeldahl Nitrogen.

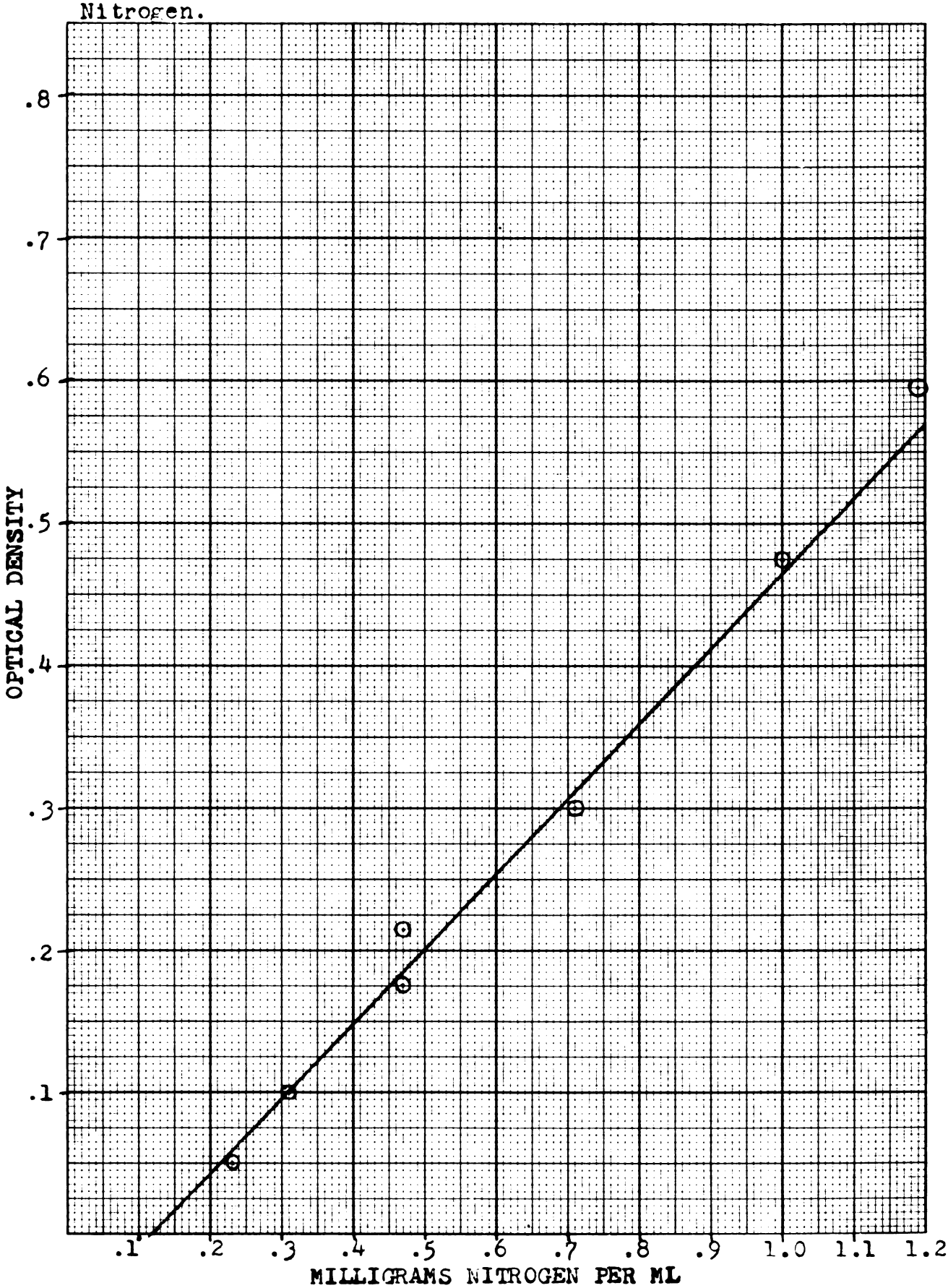


FIGURE 5. The Relationship Between Agglutination Titer and the Milligrams of Antibody Nitrogen Present

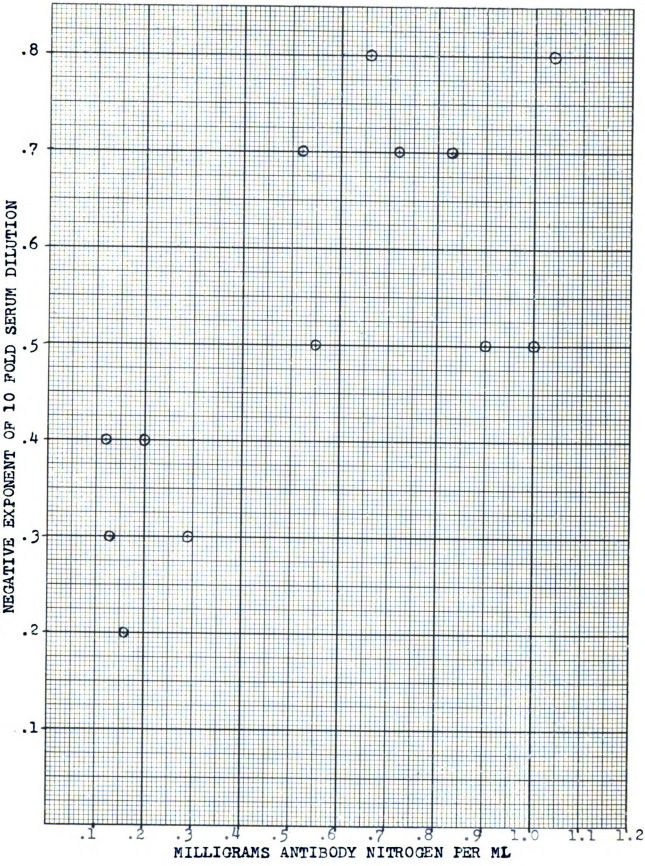


FIGURE 6. The Agglutination Titer of 2-fold Diluted Anti-Sera Related to the Respective Milligrams Antibody Nitrogen

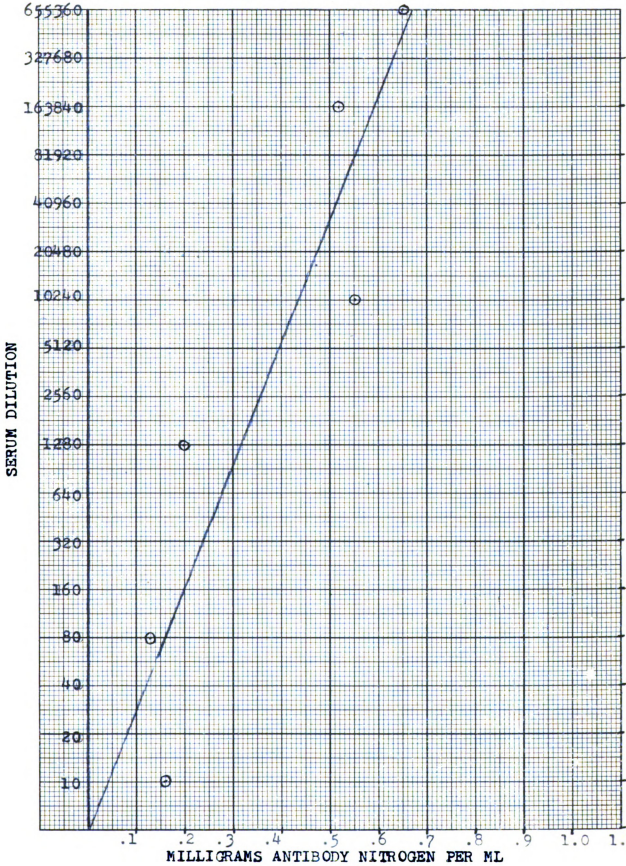


FIGURE 7. The Amount of Antibody Nitrogen Precipitated by Increasing Amounts of Antigen. Arrows Show Points at which Antibody was not detected in the Supernatant Fluid.

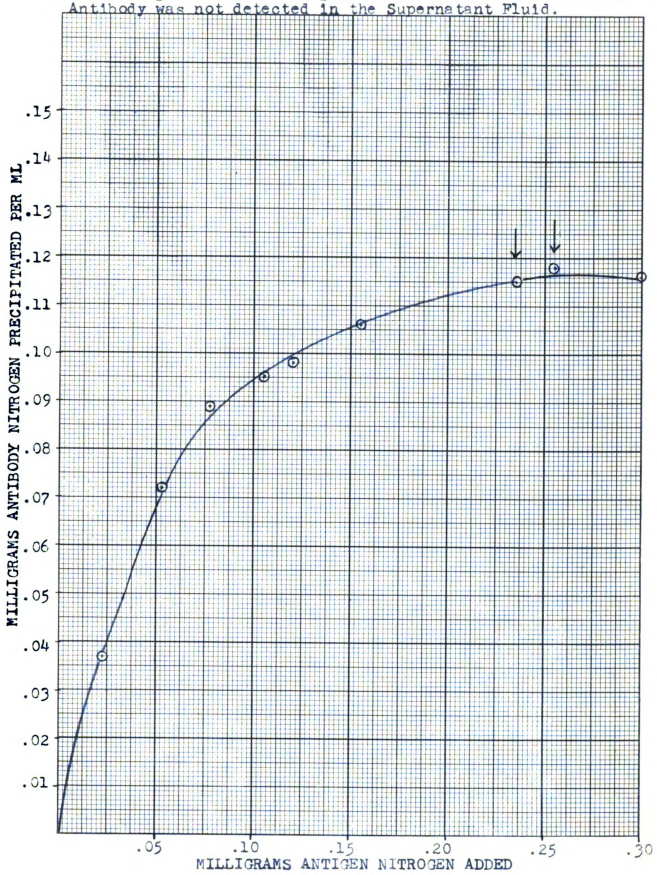
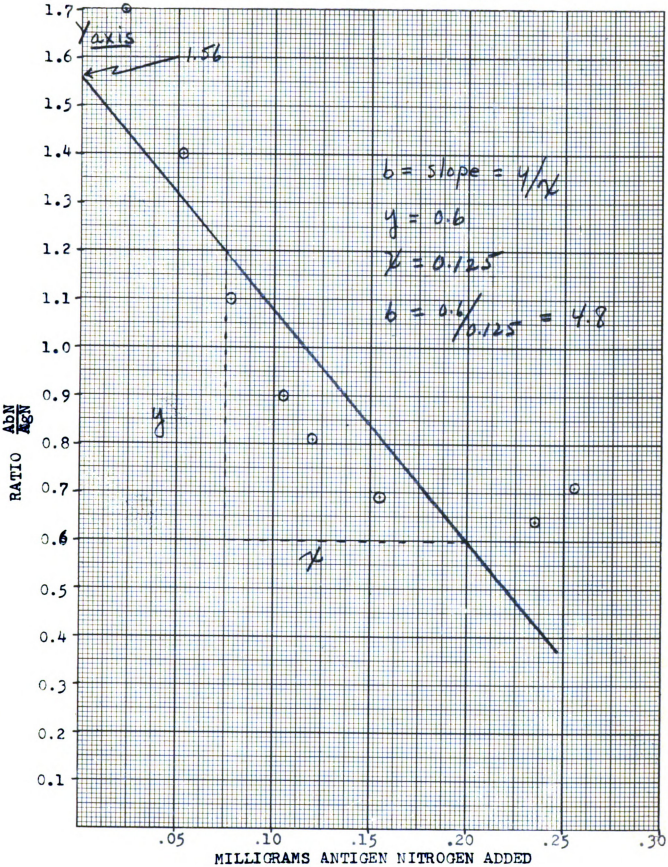


FIGURE 8. The Ratio of Antibody Nitrogen to Antigen Nitrogen Plotted Against Antigen Added



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