INVESTIGATIONS ON COMPLEMENT FIXATION IN HOG CHOLERA.

by

Adrian Jackson Durant, B. S. A.

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J. W. Connaway

TABLE OF CONTENTS.

INTRODUCTION.

At the present time no biologic test for the diagnosis of hog cholera exists by means of which the presence of the disease in a herd can be determined in the early stages of an outbreak, and before the disease has gained considerable headway. In the beginning of an outbreak, in individual herds, or in a neighborhood, time is frequently lost in awaiting developments that will furnish positive evidence of the presence of hog cholera. The importance therefore of a quick and reliable method to diagnose hog cholera is readily seen. At present the clinical symptoms, gross lesions, and the epizootic character of the disease are the only means by which we can arrive at a diagnosis. And these evidences are sometimes absent. This paper is devoted to the investigation of the possible application of the Complement Fixation Test to the diagnosis of hog cholera.

Another application of the Complement Fixation Test included in this investigation is its use as a method of testing the potency of anti-hog-cholera serum. The need of a delicate laboratory test is apparent to all workers in hog cholera serum production who are familiar with the present crude and expensive methods.

The Complement Fixation Test, for the diagnosis

of disease, was first successfully applied by Wassermann[1] in the diagnosis of syphilis in man. The principle comes from the experiment of Bordet[2], which is usually spoken of as the "Bordet-Gengou phenomenon" and is used in determining whether a given serum possesses certain amboceptors.[3]

Since 1906 the time of Wassermann's publication on this test, its use as a diagnostic agent has been extended to the following diseases of man:- Gonococcus infection, Glanders, Streptococcus infection, Pertusis, Meningitis, and Typhoid. Besides this it is also used to diagnose several diseases of domestic animals such as Contagious Abortion in cattle, Dourine and Glanders in horses.

In view of the fact that the success or failure of the Complement Fixation Test, as applied to hog cholera, depends almost entirely on the preparation of the different components used in Complement Fixation, considerable space will be given to a discussion of the phenomena of the test, and the work done by the various investigators, followed by the actual technic of the test as applied in this particular disease. The resume of this subject below was taken from Bolduan's Immune Sera.

Notes:-- 1. Wassermann, Neisser and Bruck, Deutsche med. Wochenscher, 1906. Wassermann and Plaut. Ibid. (Bolduan, Immune Sera, 1908).
2. Bordet and Gengou, Annal. Inst. Pasteur Vol. 15, 1901 (Bolduan Immune Sera).
3. Immune bodies or receptors of Ehrlich.

HISTORICAL.

BACTERIOLYSIN AND HEMOLYSIN.

As far back as 1874 Gscheidlen & Traube[1] demonstrated that considerable quantities of septic material could be injected into the circulation of warm-blooded animals without apparently any effect on the animal. Ten years later (1886) we find a similar observation made by Fodor[2]. In 1888 Nuttall[3] showed that normal blood serum possessed marked germicidal properties. A number of workers then undertook to determine the conditions most favorable to the exhibition of this phenomenon, and further to decide upon the constituent of the serum to which this property was due or whether it was a function of the serum as a whole.

In 1889 Buchner[4] published a series of experiments and showed that an exposure to a temperature 55°C. robs the serum of its bactericidal property. He also concluded that the active element in the process was a "living albumin" and suggested for its name "alexin" (complement).

Notes:-- 1. Gscheidlen & Traube Schlez. Gesellschaft. f. Vaterland. Cultur. Med. Sect., 1874.
2. Fodor, Deutsche Med. Wochenschr. 1886.
3. Nuttall, Zeitschr. f. Hygiene. Vol. IV. 1888.
4. Buchner, Centralblatt Bacteriologie. Vol. V. 1889. Archiv. f. Hygiene. Vol. X. 1890.

He found that it was possible to increase the bactericidal action (i.e. he thought the quantity of "alexin" was increased) for a particular bacterium by immunizing an animal with that bacterium.

PFEIFFER'S[1] PHENOMENON.

An enormous advance in the study of immunity was made in the discovery of Pfeiffer's phenomenon in 1894, and it is to Pfeiffer's observations that we owe the first and most important insight into the mode of action of the bacteriolytic immune sera. A normal guinea pig is able to withstand and dissolve a number of living Asiatic cholera bacilli, if these are injected intraperitoneally. If in such an animal we gradually increase the dose injected, it will be possible after a time to inject at one dose an amount of cholera bacilli that represents many times an ordinary fatal dose. If from this animal we now withdraw serum and inject it into another animal, we find that this serum, even in such small amounts as the fractional part of a centigram or even of a milligram, is able to protect the second animal against living cholera bacilli. Under the influence of these small amounts of serum of the treated animal, the organism of the untreated animal is able to dissolve large amounts of cholera bacilli, amounts which would otherwise be invariably fatal. This

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Notes:-- 1. R. Pfeiffer, Zeitschr Hygiene. Vol. XVIII. 1894.

process, as R. Pfeiffer showed, is a specific one, i.e., the serum of the guinea pig treated with cholera bacilli possesses an increased solvent power only for cholera bacilli, but not for any other species of bacteria. The active substance of such a bacteriolytic immune serum Pfeiffer called a "specific bactericide". If we allow some of this specific cholera immune serum to remain for some time (about three days or less) outside of the body, it will lose its power. If we add to this immune serum some serum of a normal, untreated guinea pig, as Metchnikoff first did, this immune serum has now acquired the power to rapidly dissolve cholera bacilli even in a test tube. Bordet[1], in 1895, showed that in order for the specific immune serum to dissolve bacilli in a test tube, it is unnecessary to add fresh normal serum; but that immune serum freshly drawn from the vein is able even under these circumstances to dissolve the bacilli.

HEMOLYSIS.

If we go back to the time when blood transfusion was first practised we find it stated that the bloods of different animals transfused into man were more or less directly injurious, and not capable of replacing human blood for this purpose. Landois[2] in a study published

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Notes:-- 1. Bordet, Annal. Inst. Pasteur, 1895(M.S.U. Library).
2. Landois, Zur Lehre von der Bluttransfusion, Leipzig, 1875.

in 1875 showed that while transfusion of a foreign blood might prove fatal to an animal the transfusion from a closely related species produced no ill effects. In 1898 Belfanti and Carbone[1] showed that if horses were injected with red blood cells of rabbits, the serum there after obtained from the horses would have acquired an appreciable toxicity for rabbits. Shortly after this, Bordet published a very interesting series of experiments. He showed that the serum of guinea pigs after these had been injected several times with 3 to 5 c.c. of defibrinated rabbit's blood acquires the property to dissolve rapidly and intensely, in a test-tube, the red blood cells of a rabbit; whereas the serum of a normal guinea pig is incapable of doing this, or does it in only a slight degree. He also showed that this action is a specific one, i.e., the serum of animals treated with rabbit blood acquires this dissolving property only for the red cells of rabbits not for those of any other species of animal. For the latter, such a serum is no more strongly solvent than the serum of a normal animal. The same property that Bordet had demonstrated could now be shown for the sera of all animal species treated with blood cells of a different species. This can be formulated as follows: The serum of animals, species A, after these have been injected either subcutaneously, intraperitoneally, or intravenously with

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Notes:-- 1. Belfanti & Carbone, Gion. della R. Acad. di Med. di Torino, 1898.

erythrocytes of species B, acquires an increased solvent action for erythrocytes of species B, and only for this species. It is therefore a specific action, and is called hemolysis and the substances which effect the solution of the red cells, hemolysins or hemotoxins.

At about the same time, and independently of Bordet, similar experiments with similar results were published by Landsteiner[1] and V. Dungern[2]. As a result of this work, the acquired toxicity of horse serum, found by Belfanti & Carbone when they treated horses with red cells of rabbits, was explained. The serum of the horses so treated had become hemolytic for rabbit blood, and therefore caused a solution or destruction of the red cells in the living body just as it did in a test-tube.

NATURE OF HEMOLYTIC SERA.

In a subsequent study Bordet[3] was able to show that the solvent power of the specific hemolysins depended on the combined action of two constituents of the specific serum. When the fresh hemolytic serum was warmed for half an hour to 55°C., it lost its power. If to this inactive serum a very small amount of the serum of a normal guinea

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Notes:-- 1. Landsteiner, Centralblatt Bacterial Vol. XXV., 1899. (M.S.U. Library).
2. Von Dungern, Münch. Med. Wochenschrift, 1898.(M.S.U. Library).
3. Bordet, Annal. Inst. Pasteur, Vol. XII., 1898.

pig was added the full hemolytic power was restored to this inactive serum. In other words it had been re-activated by this addition.

This shows that the hemolytic action of the specific hemolytic serum depends on two substances. One of these is able to withstand heating to 55°C., and is contained only in the specific serum. The other is destroyed at the same temperature above and is contained not only in the specific hemolytic serum but also in the serum of normal untreated animals. Buchner applied the term alexins (complement) to the constituents of normal serum which were actively destructive to corpuscular elements, bacteria and other cells with which they come in contact. Bordet used the same term for that constituent of normal serum which did not withstand heating to 55°C. and which was one of the factors in the hemolytic process. The other substance, which was found only in the specific serum and which withstood heating to 55°C., he termed substance sensibilatrice (hemolysin).

According to Bordet, therefore, the substances required for hemolysis are the "substance sensibilatrice" of the specific hemolytic serum and the alexin which exists even in normal serum. The action of these two substances Bordet explains by assuming that the red cell is not vulnerable to the alexin; just as, for example, there are

certain substances that will not take a dye without the previous action of a mordant. The substance sensibilatrice plays the role of a mordant. It makes the blood-cell vulnerable to the alexin, so that the latter can attack the cells and dissolve them.

Bordet says further, that the "substance sensibilatrice" sensitizes the blood cells not only for the alexin (complement) derived from the serum of the same species as that from which it (the substance sensibilatrice) is derived, but sensitizes such cells also for the alexins of normal sera of other species. In the foregoing experiment of Bordet, the substance sensibilatrice derived from the guinea pig by treatment with rabbit blood sensitizes the red blood cells of rabbits not only for the alexin of normal guinea pig blood, but also for the alexins of other normal sera. In other words Bordet showed that the substance alexin was present in all fresh normal sera as well as the fresh sensitized serum.

In reviewing the important facts that have been discussed in the foregoing pages on Hemolysis, according to Bolduan[1], we see as follows: By means of treatment of one species of animal with the red cells of a different species, the serum of the first species acquires an uncommonly increased power to dissolve and to agglutinate

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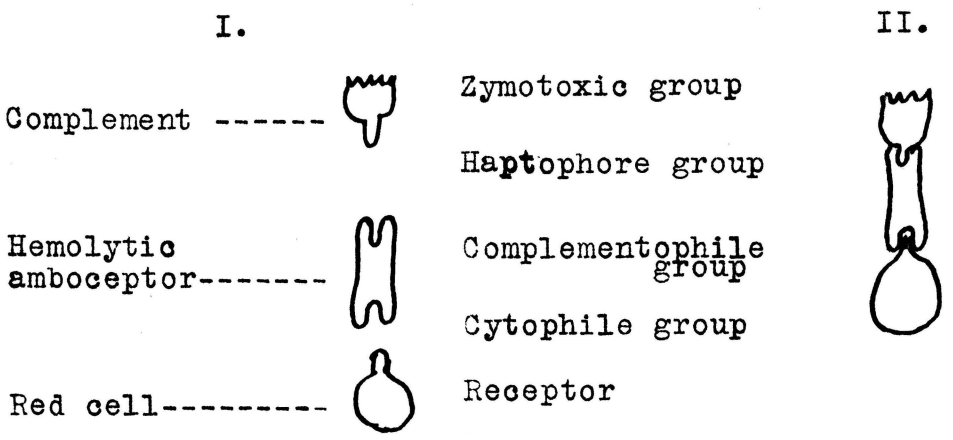
Notes:-- 1. Bolduan. Immune Sera, 1908. John Wiley & Sons.

the red cells of the second species. This increased hemolytic power shows itself not only in vivo, so that an animal so treated is able to cause red cells injected into it to rapidly dissolve and disappear, but it shows itself also in vitro when the serum of this animal is used. The process consists in the combined action of two substances that which is excited, in response to the injection, the "substance sensibilatrice", and the alexin of normal serum.

A pupil of Ehrlich, Levaditi shows in Figure 1 below the relation existing between complement, hemolytic amboceptor and red cell.

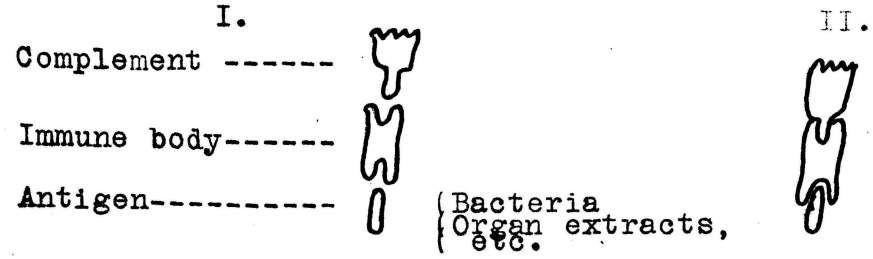
Fig. 1.



The visible phenomen/on in this experiment is the solution of the red corpuscles.

Fig. 2.

In the figure is shown the relation existing between complement, immune body and antigen.



In this experiment the bacteria are dissolved.

THE ANALOGY BETWEEN THE BACTERIOLYTIC AND HEMOLYTIC PROCESSES.

Bolduan in his Immune Sera explains the analogy as follows:-- "If we now recall the main points in (Asiatic) cholera immunity the close analogy between this and the subject of hemolysis is apparent. Just as, when immunizing an organism against cholera bacilli the organism responds with increased solvent power for those bacteria, so does the organism respond when it is treated, i.e. immunized, with the red cells of another species by increasing the solvent power of its serum for those particular cells. Furthermore, just as the hemolytic process was seen to depend on the combined action of two substances, one developed in the hemolytic serum, the other already present in normal serum, so also in the bactericidal process just studied there are two factors. It is easy to understand, therefore, what formerly was not at all clear, why specific bactericidal serum against cholera, typhoid or other infectious disease should not act in a test-tube unless there had first been added some normal serum (according to Metchnikoff), or there had been employed a perfectly fresh serum (according to Bordet): simply because in either of these ways the alexin necessary to cooperate with the "substance sensibilatrice" is introduced. This alexin no longer exists in the immune serum, if this be not perfectly fresh, for

we have seen that it decomposes either on warming or spontaneously on standing. A bactericidal serum, therefore, that has stood for some time is incapable of dissolving bacteria. It is possible, however, to make an old inactive serum again capable of dissolving bacteria in vitro by adding a little fresh alexin, according to the suggestion of Metchnikoff. In other words, it is thus reactivated. Another obscure point was cleared up by these studies: why a specific bactericidal serum which is inactive in vitro should be intensely active in the living body. This is because in the living body the serum finds the alexin necessary for its working, which is not the case in the test-tube, unless fresh normal serum be added. We see from all this that even the first experiments in hemolysis have served to clear up a number of practical points in an important branch of bacteriology".

TERMS DEFINED AND ROLE OF COMPLEMENT EXPLAINED.

In the following pages the writer will use the term immune body for the substance in bactericidal serum, and hemolytic amboceptor for the substance in hemolytic serum. Instead of the name "alexin", the term "complement" (according to Ehrlich) will be substituted expressing the idea that

this body completes the action of the immune body or hemolytic amboceptor, the complement possessing no combining group which can attach itself directly to the red blood cell or bacteria. Complement acts on these cells only through an intermediary, the immune body, which therefore must possess two binding groups one which attaches to the red blood cell and the other to the Complement of normal serum. According to Ehrlich then, the role of the immune body or hemolytic amboceptor consists in this, that it attaches itself to the red cell on the one hand and to the complement on the other, and in this way brings the digestive powers of the latter to bear upon the cell, the complement possessing no affinity for the red cell. Of course the same will hold true in the case of the bacteria and the immune body and Complement. The specific action of the hemolytic and bactericidal sera appears to be due exclusively to the immune body.

Having discussed the phenomena of bacteriolysis and hemolysis and explained the relation or analogy between the bacteriolytic and hemolytic processes by reviewing more or less briefly the work done by various investigators that go to prove these phenomena, we will now turn to the discussion of the various components used in the Complement Fixation Test and review briefly the work already done along the line of preparation of the components. As we shall see in subsequent pages, certain of these components bear an important relation to the most essential facts presented. The following discussion on the preparation of various antigens was taken from the reference given below.[1]

PREPARATION OF ANTIGENS.

As has been shown by the classical experiment of Bordet and Gengou[2] in previous pages, the reaction depends, in principle, intimately upon the fact that neither antigen alone, nor amboceptor (antibody) alone, can fix complement but that this fixation is carried out only by the combination of antigen plus amboceptor. Any specific amboceptor can be determined by this method, provided the homologous or stimulating antigen is used; and vice versa,

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Notes:-- 1. Hiss & Zinsser, Text Book of Bacteriology 1912.
2. Bordet & Gengou, Ann. de l' inst. Pasteur, XV. 1901. (Hiss & Zinsser, Text Book of Bacteriology 1912)(M.S.U.Lib.)

by the use of a known antibody a suspected antigen may be determined.

An "antigen" is a substance capable of stimulating the formation of antibodies in animals or man and as has been shown is one of the most active factors in the Complement Fixation Test.

When testing immune sera for amboceptors stimulated in man or animals by microorganisms which can be cultivated, either the whole bacteria or extracts of the bacteria may be used as an antigen, and it is also true that in the case of syphilis in man that non-specific antigens give reliable results; as we shall see in subsequent discussion.

For the diagnosis of syphilis by the Complement Fixation method, the antigen employed was originally obtained by the extraction of syphilitic organs, in which free syphilitic antigen; i.e. uncombined products of Spirochaeta pallida", were assumed to be present, as at that time pure cultures of this organism had not been isolated. It should be remembered that the test may be applied to most diseases by the substitution of suitable specific antigens. When cultivable bacteria are used as antigens, Bordet and Gengou made use of a thick salt-solution emulsion of a twenty-four-hour-agar-slant culture of the microorganism. Wassermann and Bruck[1]

Notes:-- 1. Wassermann & Bruck, Med. Klinik, 55, 1905. & Dent. Med. Woch., XII. 1906. (Hiss & Zinsser, Text Book on Bacteriology 1912).

prepared their bacterial antigen in the following way: the growths of about ten agar slant cultures are emulsified in about 10 c.c. of sterile distilled water. This emulsion is shaken for twenty-four hours in a shaking apparatus. At the end of this time 0.5 per cent of carbolic acid is added and the fluid cleared by centrifugation.

In their original experiments, Wassermann and his collaborators made use of salt-solution extracts of the organs (chiefly of the spleen) of a syphilitic fetus for the antigen to test for antibodies. This tissue substance was cut into small pieces and to one part by weight of this substance, four parts of normal salt-solution and 0.5% of carbolic acid was added. This mixture was shaken in a shaking apparatus for twenty-four hours, and after this the coarser particles were removed by centrifugation. The reddish supernatant fluid was used as the antigen and could be preserved for a long time in dark bottles in the ice chest.

Michaelis[1] obtained the antigen in the following way: The liver of a syphilitic fetus was preserved in a frozen state and from time to time small quantities of extract were prepared for the purpose of obtaining antigen. This was obtained by thoroughly grinding up a small piece of the liver in a mortar and adding five parts of salt solution and about 0.5 per cent of carbolic acid. This mixture was shaken in a shaking apparatus for several hours

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Notes:-- 1. Michaelis, Berl. klin. Woch., 1907.

and was then allowed to stand at a temperature slightly above 0°C. for several days. Finally it was cleared by filtration or centrifugation.

Alcoholic extracts of syphilitic organs have been used by a number of authors. Porges and Meier[1] extract the chopped-up syphilitic liver for twenty-four hours with five times the volume of absolute alcohol. This is then filtered through paper and the alcohol evaporated in vacuo at a temperature not above 40°C. The greenish sticky residue should have an alkaline or neutral reaction. About 1 gram of this material is then emulsified in 100 c.c. of salt solution to which 0.5 per cent of carbolic acid has been added. The fine emulsion which results is filtered through thin paper and the filtrate used as the antigen.

Proges and Meier, as well as a number of others, have discovered that in actual practice it is not necessary to make use of syphilitic organs in order to obtain an antigen which will combine with syphilitic immune body. This fact, of course, has thrown much suspicion upon the specificity of the phenomenon. In practice, however, it appears as a purely empirical fact that many of the non-specific antigens, nevertheless, give reasonably reliable results. The authors mentioned above have found that a 1 per cent emulsion of commercial lecithin (Kahlbaum) in carbolized salt solution furnishes a suitable antigen.

Notes:--1. Porges & Meier, Berl. klin. Woch., XV. 1908.

This has not been universally confirmed. The same authors have obtained good results by extracting a normal fetal liver by alcohol in the same way as they extracted the syphilitic organ. Landsteiner, Muller, and Poetzl[1] have successfully employed an alcoholic extract of the heart substance of a guinea-pig.

Similar alcoholic extracts of normal human spleen or of normal rabbit's liver may be employed. Although often claimed that the antigen in such extracts is furnished by the lipoids, as a matter of fact it is at the present day unknown to which ingredient the immune-body binding power is to be attributed.

Hiss had good results with the following:- Fresh normal liver or spleen is covered and thoroughly macerated with five times its volume of absolute alcohol. This is allowed to extract in the incubator for six to eight days, being thoroughly stirred up at least once a day. It is then pressed through cheese-cloth and filtered through paper. This alcoholic extract is evaporated to dryness at room temperature with the aid of a wind fan. The sticky, brownish residue resulting is taken up in a small quantity of ether and the solution poured into four times its volume of C. P. acetone. A heavy flocculent precipitate

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Notes:-- 1. Landsteiner, Muller & Poetzl, Wien. klin. Woch., 50, 1907.

forms which settles to the bottom as a sticky brown mass. This is retained as antigen and may be preserved under acetone. The acetone-soluble fraction is thrown away. For use, about 0.2 gram of the sticky paste is dissolved in about 5 c.c. of ether and 100 c.c. of salt solution added. This is shaken until the ether has evaporated. The resultant antigen, ready for use, is a slightly opalescent greenish fluid from which nothing settles out on standing.

Antigen for diagnosing Dourine in horses is prepared from the spleen of guinea pigs which are inoculated with the specific organism and allowed to die. The spleens are then ground up finely, preservative added and extracted with salt solution by shaking 12 hours in shaking machine. It is then filtered and is ready for titration.

Before an antigen can be used for the actual test, it is necessary to determine the quantity which will give accurate results. However, since the principle for the adjustment of this reaction is practically the same for all antigens, the writer will at this point take up the discussion of the production of antigen, and other components used in Complement Fixation, as applied to hog cholera, and give in detail the actual technic in every case.

APPLICATION OF THE FOREGOING PRINCIPLES AND METHODS TO HOG CHOLERA.

Hog cholera is a highly infectious and highly fatal disease of unknown etiology. The micro-organism is ultra microscopic and filterable and has not been cultivated artificially. The difficulties therefore of applying the Complement Fixation Test would seem to be well nigh unsurmountable. On the other hand this is an immunizing disease. Hogs that recover from a natural attack are as a rule permanently immune. They must therefore produce immune bodies in response to the stimulation of an active virus. Moreover, the "immune bodies" are utilized in a practical way for the protection of susceptible hogs by the injection of small quantities of the blood of hyperimmunized swine. Viewing the problem from this side the difficulties do not seem so great. It is simply a matter of preparing the "antigen" which is certainly abundant in some animals and preparing the antibodies, which are certainly abundant, in a suitable form to show the reaction.

~~In the~~ preceding pages it has been shown that it is possible to secure an antigen specific for certain diseases without having the specific organism isolated

and grown in pure culture. This fact is well illustrated in the case of the syphilitic antigen which was secured in several different ways; and in the diagnosis of that particular disease it was found that it was not essential that the specific organism be present even for the production of an antigen; in other words it was found that a non-specific antigen would deviate the complement when in the presence of specific syphilitic antibodies. The reaction was found to be specific however, in view of the fact that a deviation would occur only when the serum or spinal fluid was of syphilitic origin. (According to Landsteiner, Muller & Poetzl the serum of animals infected with dourine also gives rise to inhibition of haemolysis when tested according to the above method).

The method of preparation of an antigen for the diagnosis of Dourine by the Complement Fixation Test illustrates again that a suitable antigen can be secured from organs of an infected experiment animal of another species and gives reliable results.

The course pursued in the investigations of the diagnosis of hog cholera by means of the Complement Fixation Test was to try to secure an antigen from extracts of the serum, spleen, kidneys, or liver of hogs with acute symptoms of hog cholera. It was thought

that these materials would probably furnish an antigen; since the blood in the acute type of cholera possesses a high degree of virulence and the organs mentioned often show marked lesions.

The clinical history and post mortem findings of the pigs used in this work for the production of the antigens are appended. It will be noted that they all showed the usual lesions and clinical picture of hog cholera in the acute form.

METHOD OF PREPARATION OF ANTIGEN EXTRACTS.

Half the pigs used in these experiments were artificially inoculated with 2 c.c. of virus and were killed when showing the most severe symptoms of hog cholera. The other half were naturally infected. (See post mortem records - Pigs No. 1 to 8.)

PIG NO. 1.

This pig was killed and the spleen and kidneys were secured under aseptic conditions. The spleen was ground finely in a meat chopper; and salt solution was added four parts to one part of spleen pulp. The mixture was then shaken in shaking machine for 12 hours,

then filtered and 10% of Glycero-Carbol-salt solution[1] was added as a preservative.

The kidney was treated in the same way as the spleen. For titration of these two extracts see Table III.

PIGS NO. 2, 3, & 4.

These pigs were killed and the spleens and kidneys were secured in the same manner as described for Pig. 1 except that the three spleens were ground up together and a composite sample was taken to be extracted. The kidneys were treated in the same way, a composite sample was taken to be extracted by shaking with salt solution. For titration see Table III.

PIG NO. 5.

This pig was one which was secured from a farm where a number had already died with acute symptoms of cholera. It was dead when it reached the laboratory. From the post mortem record of this pig, it will be seen that it showed an acute haemorrhagic type of cholera. The spleen and kidneys were treated in the same manner as described for Pig No. 1.

It will be noted that the spleen of this pig furnished an active antigen extract. For titration see Table III.

Notes:-- 1. Glycero-Carbol Salt Solution, normal Saline 85%; Glycerin 10%; Carbolic acid 5%.

PIGS NO. 6 & 7.

These two pigs, 30 pounds in weight, were secured from the same farm from which No. 5 came, and were alive when brought to the laboratory. They were both killed by etherizing, a composite sample of the two spleens and another composite sample of the four kidneys were treated in the same manner as described for Pig No. 2, 3, and 4. For titration see Table III. Neither of the samples furnished an antigen.

PIG NO. 8.

This pig was one which died a few hours after it was born. It showed on post mortem the hemorrhagic lesions common to hog cholera, though it came from a sow which had been vaccinated against cholera and which on slaughter two days later showed no lesions of cholera. The liver was secured from this pig, ground finely and extracted in the usual way, by adding one part of liver pulp to four parts of salt solution, then shaking in a shaking machine for 12 hours. Titration for antigen negative. See Table III.

Since there was doubt as to whether the lesions in this pig were due to cholera some of its blood was injected into two pigs that were supposed to be susceptible. The results were negative.

Note:- Some of the work planned could not be completed before the time limit set for handing this Thesis; as "Test of a number of liver extracts for antigen". This will be done later.

PREPARATION OF COMPLEMENT.

Necessarily the preparation of the different components for this test would be the same, for hog cholera, as in various other diseases, since the only difference in the various tests lies in the antigen, as will be seen in the subsequent discussion. The complement used in this work was secured in all cases from healthy guinea pigs. Guinea pigs being best adapted to furnish complement on account of the more constant and very active complemental qualities of their serum. Only a small quantity is required in conjunction with the hemolysin to bring about hemolysis. In securing the blood, the guinea pig was etherized, and a transverse incision was made across one side of the neck with a sharp scapel, cutting the jugular but not the carotid, trachea, or oesophagus. The blood was allowed to flow into a sterile Petri dish which was set into an incubator for 15 to 20 minutes (or it can be put in the ice-box over night if not required for immediate use). After the serum separated from the clot it was drawn off with a pippette and then centrifuged in order to throw down the few red cells or other solids that were present in the serum. The clear supernatant serum was then titrated as per Table IV.

Complement being a thermolabile substance is

naturally very sensitive to external influences. Wall[1] states that complement will remain active for three days, and sometimes longer. My experience shows that complement will keep for two days and be reliable, but the titre should always be taken and adjusted on the same day it is used, as it will some times suddenly lose its activity. Needless to say that it should always be kept at a low temperature; on ice.

(The above described method of securing the blood from the guinea pig was found by the writer to be very good from an economical standpoint in view of the fact that one guinea pig could be used two or three times to furnish serum for the complement if sufficient time was allowed to elapse between bleeding. By simply severing the jugular vein the guinea pig is not killed, and at the next bleeding can be bled from the other side of the neck. The third time however it is generally necessary to cut both the jugular and carotid thereby killing the animal). (Another method that may be employed is to aspirate the blood from the heart of the guinea pig with a hypodermic syringe, using a small aspirating needle).

Notes:-- 1. Wall, S. Uber die Feststellung des seuchenhoften Abortus beim Rinde durch Agglutination & Komplementbindung. Zeitschrift fur Infectionskrankheiten der Houstiere. Band 10, Heft $\frac{1}{2}$ /3. 1911.

THE PREPARATION OF HEMOLYSIN.

The phenomenon of hemolysis has already been described in previous pages and is simply the power which the blood serum of an animal of one species acquires to dissolve the red blood corpuscles of an animal of another species, when injected with such corpuscles. In the process of dissolution, the hemoglobin or red coloring matter of the corpuscles is liberated. It is upon this fact that the utilization of the Complement Fixation as a diagnositc agent is based.

For the production of hemolysin in this work, a rabbit was immunized against horse corpuscles. But it was found that the serum of hogs was highly hemolytic for the red cells of this horse (No. 1); it was also found hemolytic for another horse (No. 2), and for a goat, and for two cows (Nos. 1 and 2). Finally a cow (No. 3) furnished red cells, for which the serum of hogs was not hemolytic. It was then necessary to sensitize another rabbit against the corpuscles of cow (No. 3), as it can easily be seen that it is necessary to have red cells which would not be hemolized by the hog serum in order to carry out any investigation dealing with hog cholera. The procedure to be followed in procuring and washing the cow blood corpuscles will be found under "The Washed Red Blood Cow Corpuscles."

Equal parts of the washed corpuscles and salt solution were mixed and 14 c.c. of this suspension were injected intraperitoneally; at the end of seven days another injection was given, 20 c.c. were administered, and after another seven days 30 c.c. more of the washed corpuscles were injected.

Seven days after the last injection a sample of blood was taken from an ear vein of the rabbit for examination. This blood serum was found to be hemolytic for the cow corpuscles, as expected.

The titre of this rabbit serum or hemolysin was next established,-- See Table No. V. As the preliminary test of the rabbit's serum proved to possess sufficient hemolytic power for use, the rabbit was subjected to a further bleeding. The blood was drawn from the anterior ear vein into a sterile centrifuge tube. The rabbit was not killed but was kept for further use to furnish serum by additional injections with cow corpuscles. One subsequent injection will usually stimulate a further production of hemolytic bodies. Before bleeding, the ear of the rabbit was shaved and carefully disinfected with alcohol, and all the precautions of asepsis observed. The blood was allowed to clot and the clot was loosened from the sides of the test tube with a sterile scapel. This tube was then placed in a centrifuge machine and the serum was separated from the clot, poured off into a sterile

bottle and heated 56°C. for 20 minutes in order to "inactivate" it. It was then stored in the ice-box. The titre was re-established every two weeks, as the serum in some cases has been found to lose its activity quite rapidly after storage. Although the writer has found that if hemolysin is kept at a low temperature, without preservative, it will retain its hemolytic properties for four or five months.

THE WASHED RED BLOOD CORPUSCLES.

The red blood corpuscles of cow No. 3 were employed in the preparation of the hemolysin, also as an essential factor in the test proper. The reason for using the red cells of cow No. 3 for this investigation is explained under "Preparation of Hemolysin".

To obtain the blood from the cow, to furnish the red cells, a cord was passed around the neck close to the shoulder and drawn tightly. A hypodermic needle was then thrust into the engorged jugular vein, and the blood was caught in a sterile bottle containing glass beads. The blood was defibrinated by shaking the bottle for ten minutes after which it was filtered through sterile gauze into centifuge tubes. These were placed into a centrifuge machine, with a speed of 2000-3000 revolutions per minute, until all of the corpuscles were thrown down. The supernatant serum was pipetted off and a corresponding amount of sterile physiological salt solution added to

remove the remaining traces of serum, a very necessary procedure, after the last washing the salt solution was drawn off with a pipette. Two c.c. of the concentrated red cells were mixed with 98 c.c. of salt solution making a two per cent suspension of red cells. 0.5 c.c. of this two per cent suspension of red cells were used as a base from which all the titrations were established. If the red cell suspension is kept on ice it can be used safely for a week. Any evidence of red coloring in the supernatant salt solution after the red cells have settled to the bottom of the retainer is an indication of disintegration of red cells and the solution should be discarded and a fresh suspension made up.

TITRATION OF ANTIGENS.

In testing the extracts for antigenic properties in all of these preparations, the serums of hyper-immune hogs were used as it was assumed that these serums would contain a high antibody content, specific for the antigen sought for in these extracts. This is a natural conclusion since the serum of hyper-immunes when injected into a susceptible hog will protect this hog against cholera.

Susceptible pigs' serum was used as a control in every case as shown in Table II. again assuming that this serum would be negative or at least very low in

antibody content specific for cholera.

Of course, the chief object in the titration of an antigen extract would be to determine the smallest quantity of an antigen which would prevent hemolysis when the other factors of the Complement Fixation Test were present in proper quantities.

However, in this case we have an extract which we wish to examine for antigenic properties, and is really a case, or we will assume so at least, of having a known antibody to determine a suspected antigen.

The procedure followed in this titration is shown in Table No. II., Figure No. 3. A series of twelve small test tubes were arranged as in previously described titrations, and the different fluids were added as indicated. It will be observed that an incubation was required to bring about the desired reaction (provided of course the antigen was active) before the hemolytic system was added.

After a further incubation of 45 minutes the results were read according to the degree of hemolysis. (See Figure No. 3 & Table No. II.). Some one of the first seven tubes (all of which contain hyperimmune hog serum, and varying amounts of antigen (extract No. 2) 0.01 to 0.3 with the other components) will indicate the titre, i.e., the first one of the series which shows no hemolysis. In

the illustration (Figure No. 3) tube 2 containing .02 c.c. represent the titre. Tube 8 which contains serum from a pig susceptible to cholera shows complete hemolysis, because the susceptible serum does not contain antibodies specific for the antigen. Tubes 9, 10, 11, and 12 contain antigen (extract No. 2) and the hemolytic system to demonstrate that the antigen in each, without the presence of a serum containing the specific antibodies do not prevent hemolysis.

In the illustration of the titration the results given are those obtained from a positive reacting extract, to a particular hog serum. A more lengthy discussion of this which will be given at another point in this paper.

PREPARATION AND TITRATION OF SERUM OF CHOLERA HOGS TO TEST FOR ANTIGENIC PROPERTIES.

The serum from cholera hogs No. 1, 2, 3, 4 and 5, which furnished the spleen and kidney extracts already considered was also tested for antigenic properties. The blood was drawn from pigs No. 1, 2, 3 and 4 when killed. Pig No. 5 had been dead about 3 hours when the blood was drawn. The blood was defibrinated, and the red cells separated by centrifugation, and samples of the serum from each pig was tested separately. The

serum of each pig was titrated as an antigen and the technic followed was the same as shown for the other extracts, Table No. II. Figure No.3 . It was assumed in this test that the serum being tested for antigenic properties would also contain antibodies in sufficient quantities to deviate the Complement consequently no immune serum was added. The results of the titrations are given in Table I. . following:-

TABLE NO. I.

RESULTS OF TITRATION OF VIRUS

FOR ANTIGENIC PROPERTIES.

Pig No. 1.	Titration results.
(a) Serum, unheated and no preservative added.	Negative.
(b) Serum, plus 10% Glycero-carbosalt sol. heated 56°C. 20 min.	Negative.
(c) Serum, plus 10% Glycero-carbosalt sol. heated 56°C. 20 min.	Negative.
(d) Serum, plus 10% Glycero-carbosalt sol. heated 56°C. 20 min.	Negative.

Pig No. 2.	Titration results.
(a) Raw unheated serum plus preservative	Negative.
(b) " " " " " "	"
(c) " " " " " "	"
(d) " " " " " "	"
(e) " " " " " "	"

Pig No. 3

	Titration results.
(a) Raw serum unheated	Negative.
(b) Serum heated 56°C. 30 min.	"
(c) Serum 10% Carbo-glycero-salt solution	"

Pig No. 4.

	Titration results.
(a) Raw serum unheated	Negative.
(b) Serum heated 56°C. 30 min.	"
(c) Serum 10% Carbo-Glycero-salt solution	"

Pig No. 5.

	Titration results.
(a) Raw serum unheated	Negative.
(b) Serum heated 56°C. 30 min.	"
(c) Serum 10% Carbo-Glycero-salt solution	"

It will be observed from the data presented that the results were all negative; however there are several points that are open for criticism in this investigation, and it may be that by a slight change in the technic positive results may be obtained.

Perhaps by a more delicate adjustment of the components used or a slight change in the preparation of the extract of the serum success may yet be attained from the use of the serum of cholera hogs to furnish a suitable antigen. If not the serum perhaps the blood clot will furnish an active antigen extract.

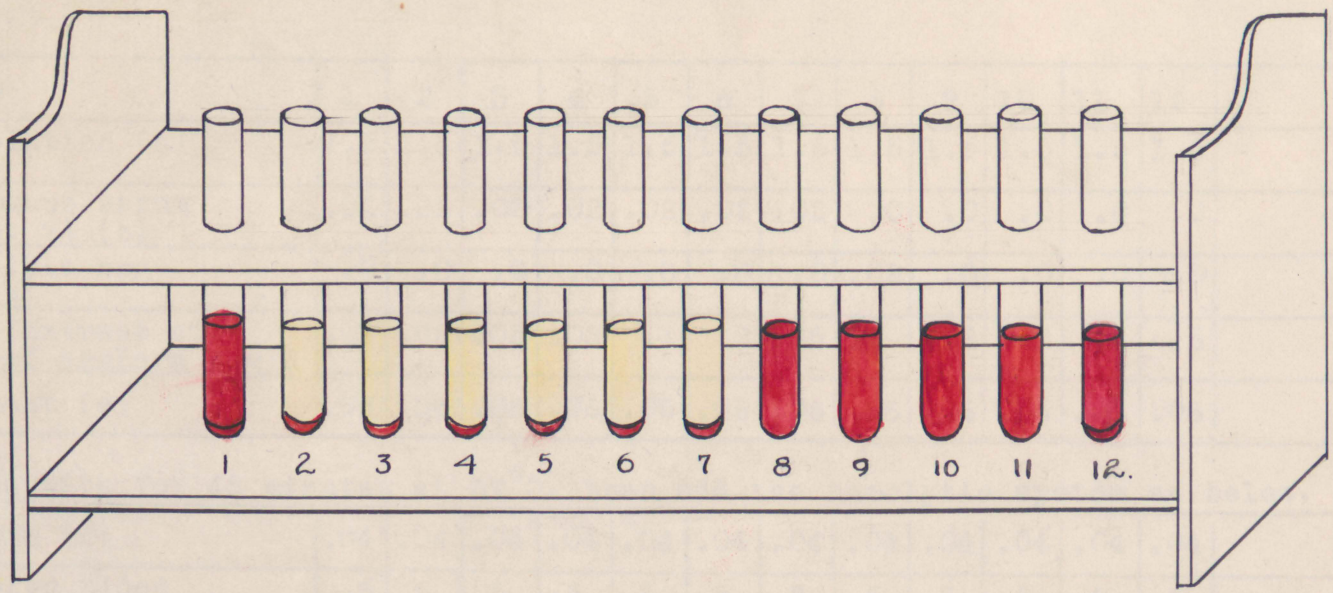

Figure 3. Titration of The Antigen.

see Table opposite

TABLE NO. II.

TITRATION OF ANTIGEN EXTRACT.

Tube	1	2	3	4	5	6	7	8	9	10	11	12
Nacl solution (a)	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Hyperimmune serum (Hog No. 7) (b)	.02	.02	.02	.02	.02	.02	.02	.0	.0	.0	.0	.0
Susceptible hog serum (c)	.0	.0	.0	.0	.0	.0	.0	.02	.0	.0	.0	.0
Antigen(Extract of spleen of cholera hog)d	.01	.02	.05	.08	.1	.2	.25	.1	.2	.3	.4	.5
Complement (e)	.06	.06	.06	.06	.06	.06	.06	.06	.06	.06	.06	.06
Incubate for 45 minutes at 37°C. then add the hemolytic system as below.												
Hemolysin (f)	.04	.04	.04	.04	.04	.04	.04	.04	.04	.04	.04	.04
Suspension blood corpuscles. (g)	.5	.5	.5	.5	.5	.5	.5	.5	.5	.5	.5	.5
Results after 45 minutes incubation. (h)	+	—	—	—	—	—	—	+	+	+	+	+

a. 0.875% sodium chloride solution.
b. Hyperimmune serum (Hog No.7) supposed to contain antibodies.
c. Susceptible hog serum not containing antibodies.
d. Antigen (extract of spleen of cholera hog).
e. Complement of known titre diluted 1.5 times.
f. Hemolysin of known titre two units used.
g. 2% suspension washed cow-blood corpuscles in sodium chloride solution.
h. + sign indicates complete hemolysis. — sign indicates no hemolysis.

TABLE NO. III.

RESULTS OF TITRATION OF THE SPLEEN AND KIDNEY ANTIGEN EXTRACTS.

		Titration Results.
1	Spleen extract from Pig No. 1	Negative.
2	" " " " Nos. 2,3,& 4	Positive. +
3	" " " " No. 5	Positive. +
4	" " " " Nos. 6 & 7	Negative.
5	Kidney extract from Pig No. 1	Negative.
6	" " " " Nos. 2,3,4	Negative.
7	" " " " No. 5	Negative.
8	" " " " No. 6 & 7	Negative.
9	Liver extract from Pig. No. 8	Negative.

It will be observed from the above table that out of 9 extracts titrated two showed active antigenic properties. Positive extract No. 2 coming from Pigs Nos. 2, 3, & 4 which were artificially inoculated with 2 c.c. of virus and were killed when showing the most acute symptoms. Positive extract No. 3 on the contrary came from Pig No. 5 which was naturally infected on a farm and the spleen was taken for extraction after the pig had been dead from the effects of cholera for about three hours.

TITRATION OF COMPLEMENT.

The technic of this titration is the same as that used by Hadley & Beach[1] in preparing complement for the Complement Fixation Test for Contagious Abortion only that the writer uses a 2% suspension of the cow-blood cells instead of a 1% suspension of the horse-blood cells, since it was found that the erythrocytes of the horse were hemolyzed by hog serum. It is necessary to establish the smallest amount of complement which with a definite quantity of hemolysin, will induce a complete hemolysis of 0.5 c.c. of a 2% suspension of the cow-blood cells in salt solution. Care must be observed in all these titrations that the exact amounts of the different fluids are used, and that dilution are made according to directions.

The complement is diluted 1.5 times with salt solution. One tenth cubic centimeter of hemolysin is assumed to represent the quantity of this substance required. Seven test tubes of about 6 c.c. capacity are arranged in a rack and into each are carefully measured different amounts of the necessary components as per Table IV. Figure 4.

It should be noted that in the titration of the different components, the results are subject to a great

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Notes:-- 1. Hadley & Beach. Research bulletin 24 Wis. Exper. Station.

deal of variation, no two titrations of different samples of the same component being exactly alike.

Shake each tube well, place the rack in the incubator at 37°C. for 45 minutes, after which a reading is made (See Figure 4). The titre of the complement is represented by the smallest quantity which completely dissolves the red corpuscles and leaves a clear, red solution. In the titration of the sample from which Figure4 was taken tube 2 represents the titre. Tubes 6 and 7 are controls; 6 should show no hemolysis as no complement is present, and shows that the hemolysin has been properly inactivated, 7 is a control for the guinea pig serum to ascertain that it does not contain hemolytic substances; all rabbit serum is excluded from this tube. Complement with a lower titre than .06 represented by tube 3, is discarded.

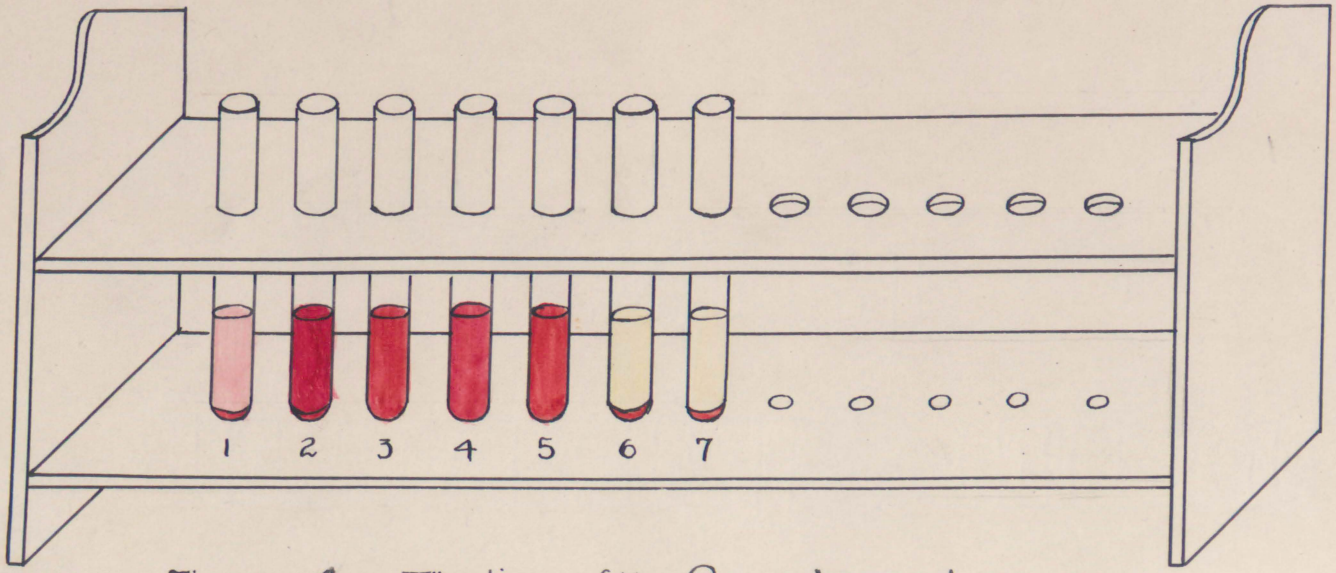

Figure 4. Titration of the Complement.

TABLE NO. IV.

TITRATION OF COMPLEMENT.

Tube	1	2	3	4	5	6	7
Nacl solution(a)	c.c. 1.5	c.c. 1.5	c.c. 1.5	c.c. 1.5	c.c. 1.5	c.c. 1.5	c.c. 1.5
Hemolysin (b)	.1	.1	.1	.1	.1	.1	.0
Suspension blood corpuscles (c)	.5	.5	.5	.5	.5	.5	.5
Complement (d)	.02	.04	.06	.08	.1	.0	.1
Result after 1 hour incubation at 37°C.(e)	∓	+	+	+	+	—	—

a. 0.875% sodium chloride solution.
b. 1% dilution hemolysin in sodium chloride solution.
c. 2% suspension washed cow-blood corpuscles in sodium chloride solution.
d. Complement diluted 1.5 times with sodium chloride solution.
e. + sign indicates complete hemolysis; — sign indicates no hemolysis; ∓ signifies a partial hemolysis in the above titration

TITRATION OF HEMOLYSIN.

The object of this titration was to establish the amount or unit of hemolysin to be used in the Complement Fixation Test. A unit of hemolysin is the smallest quantity which will bring about the complete solution of 0.5 c.c. of a 2% suspension of cow-blood corpuscles in the presence of the proper amount of complement. A series of eight test tubes were arranged. Into each was measured 1.5 c.c. of salt solution, 0.5 c.c. of the washed cow-blood corpuscles, .06 c.c. of complement then graded amounts of hemolysin were added according to Table V.

Shake each tube well, place the rack in the incubator for 45 minutes then read the results. The standard or titre of the hemolysin is, the smallest quantity which completely dissolves the red blood corpuscles and leaves a clear red solution. Hemolysis may appear in any of the first six tubes. Tube 7 is a control, with no complement to demonstrate that the hemolysin was properly inactivated and that it alone does not have a hemolytic effect. Tube 8 is likewise a control to prove that the complement without the hemolysin will not provoke hemolysis.

From one and a half to two units of hemolysin was used for all further titrations. The titre of the diluted hemolysin should

not be less than .05 which is represented in tube 3 Figure 5 . Serum with a lower titre than this was not used as the larger quantities that would be required might interfere with the reaction.

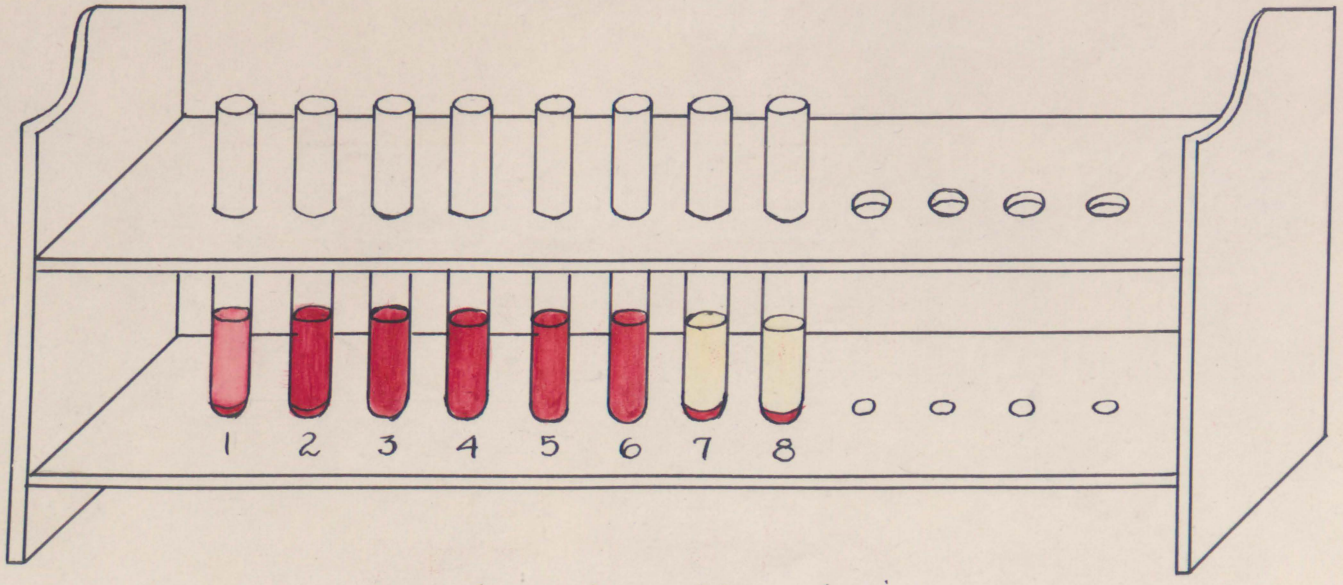

Figure 5. Titration of the Hemolysin.

TABLE NO. V.

TITRATION OF HEMOLYSIN.

Tube	1	2	3	4	5	6	7	8
Nacl solution (a)	c.c. 1.5	c.c 1.5	c.c. 1.5	c.c. 1.5	c.c. 1.5	c.c. 1.5	c.c. 1.5	c.c. 1.5
Hemolysin (b)	.02	.03	.05	.1	.15	.25	.15	0.0
Suspension blood corpuscles (c)	.5	.5	.5	.5	.5	.5	.5	.5
Complement (d)	.06	.06	.06	.06	.06	.06	0.0	.06
Result after 1 hour incubation at 37°C. (e)	∓	+	+	+	+	+	−	−

a. 0.875% sodium chloride solution.
b. 2% hemolysin in sodium chloride solution.
c. 2% suspension washed cow-blood corpuscles in sodium chloride solution.
d. Complement of known titre.
e. + sign indicates complete hemolysis; − sign indicates no hemolysis; ∓ signifies partial hemolysis.

METHOD OF PERFORMING THE COMPLEMENT FIXATION TEST IN TESTING THE SERUM OF HOGS.

In the previous pages I have described the work we have done in preparing the antigens and establishing the titre or standard of each component to be used in the Complement Fixation Test, of hog serum, for the specific immune bodies of hog cholera. The application of the test will now be considered.

In the following tests the two antigens used were secured, one from the spleens of pigs Nos. 2, 3, and 4 (composite sample), and the other from pig No. 5. (see preparation of antigen). As will be shown the serums tested were from hyperimmunes, and from hogs showing acute symptoms of hog cholera.

The test proper was carried out as follows:- a rack with five small test tubes was arranged for each animal to be tested. The required amounts of serum, antigen, and complement were measured into the respective tubes, each tube shaken well and incubated at 37°C. for 45 minutes. The hemolytic system was then added according to Table VI. . After incubating again for 45 minutes the results were read.

The purpose of the first incubation above was to give an opportunity for the complement to combine with the antigen through the medium of the immune bodies which may be present in the suspected serum being tested.

Table No. VI. on the following page shows a positive reaction and Table No VII. on the next page shows a negative reaction.

It will be observed that all the components used were the same in each test except the hog serum that was tested; the samples came from different individuals. The results indicate that the blood of one contained specific "immune bodies" for the "antigen" used; and that the blood of the other hog did not contain these immune bodies, at least in quantities that could be detected by the test.

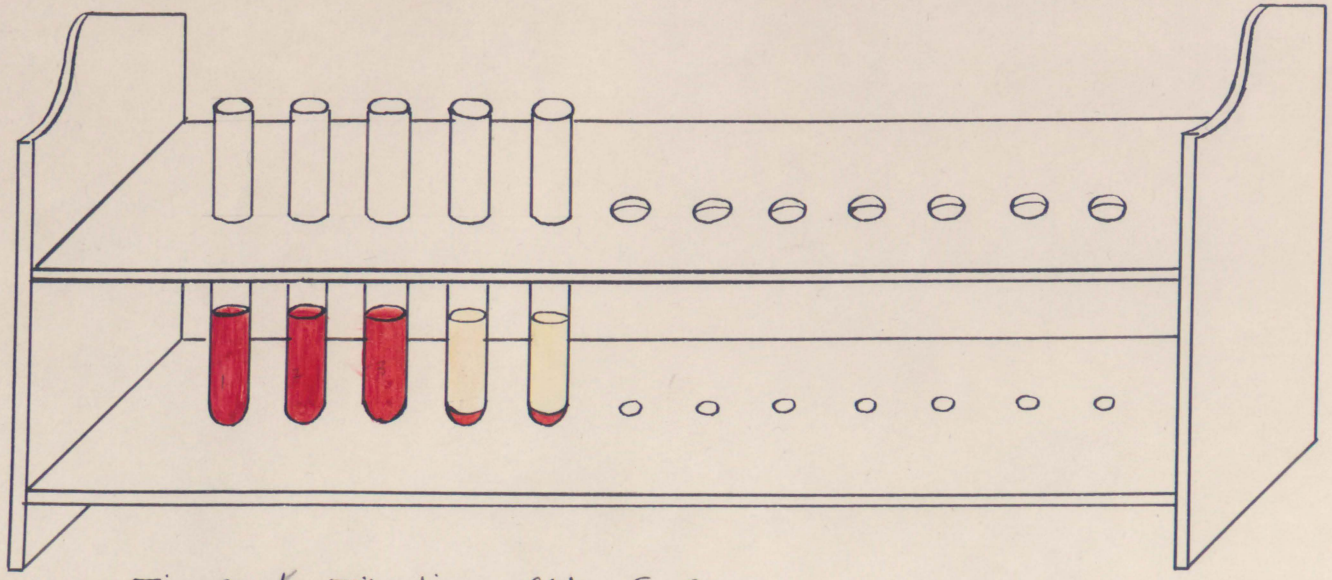

Figure 6. Titration of Hog Serum.
Postive Reaction.

TABLE NO. VI.

COMPLEMENT FIXATION TEST.

Hyperimmune hog showing positive reaction.
#7— 7

Tube No.	1	2	3	4	5
Salt Solution	c.c. 1.5	c.c. 1.5	c.c. 1.5	c.c. 1.5	c.c. 1.5
Suspect Hog Serum no 7			.02	.02	.03
Antigen(spleen extract from pig 2,3, & 4)	.04			.04	.04
Complement	.06	.06	.06	.06	.06
Incubate 37°C. for 45 minutes.					
Hemolysin	.04	.04	.04	.04	.04
Red cells	.5	.5	.5	.5	.5
Incubate 37°C. for 45 minutes.					
Results	+	+	+	—	—

+ (plus) sign indicates hemolysis.
— (minus) sign indicates no hemolysis.
∓ Indicates partial hemolysis.

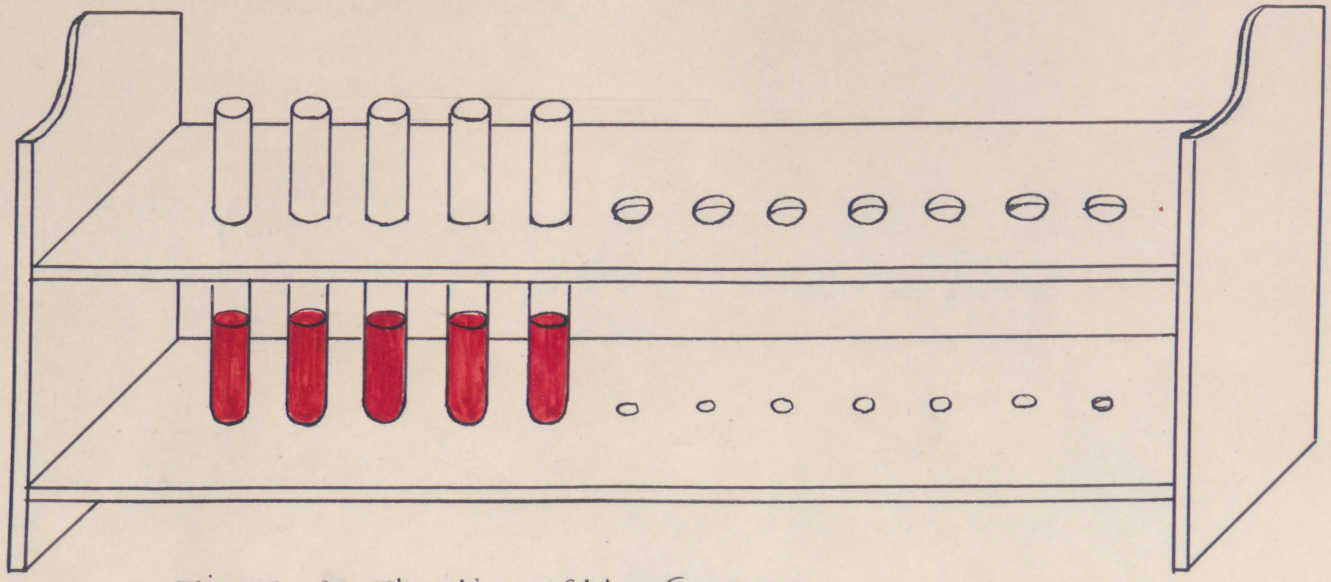

Figure 7 Titration of Hog Serum.
Negative Reaction.

To Kinder
Top face right to p47

TABLE NO. VII.

COMPLEMENT FIXATION TEST.

Hyperimmune hog showing negative reaction.

Tube	1	2	3	4	5
Salt Solution	c.c. 1.5	c.c. 1.5	c.c. 1.5	c.c. 1.5	c.c. 1.5
Suspect Hog Serum			.02	.02	.03
Antigen(spleen extract from pig 2,3, & 4)	.04			.04	.04
Complement	.06	.06	.06	.06	.06
Incubate 37°C. for 45 minutes.					
Hemolysin	.04	.04	.04	.04	.04
Red cells	.5	.5	.5	.5	.5
Incubate 37°C. for 45 minutes.					
Results	+	+	+	+	+

+ (plus) sign indicates hemolysis.
— (minus) sign indicates no hemolysis.
∓ Indicates partial hemolysis.

The results given in Table No. VI. . and Figure No. 6 was taken from a test of the serum of hog No. 7 which was positive, in the titration of antigen extract No. 2 (from spleen pulp of pigs No. 2, 3, and 4.), and antigen No. 3 from spleen of Pig No. 5.

The interpretation as shown in Table No. VI.— is the degree of hemolysis. The first three tubes are controls. Tube 1 shows hemolysis because there is no antibody present to bind the complement; this tube demonstrated that the "antigen" in the quantity used in the test did not of itself bind the complement. Hemolysis occurred in Tube 2, as the complement and hemolysin were present in the proper quantities to bring about solution of cow corpuscles. Tube 3 which contains suspected serum shows hemolysis; this indicates that the serum without the antigen does not prevent hemolysis. Tubes 4 and 5 contain all of the components and show no hemolysis indicating that the "complement" was deviated in the first incubation showing the presence of "antibodies" in the serum tested. Of course in a case where no antibodies were present complete hemolysis would occur in all tubes, as shown in Table No VII— Figure 7 .

TABLE NO. VIII.

SHOWING RESULTS OF TESTS OF

HOG SERUM WITH ANTIGEN NO. 2.

The hogs used in this work were hyperimmunes which were being used to produce anti-hog-cholera serum. The samples of blood being taken at the time of bleeding.

The samples were caught in sterile test tubes, and the serum was separated from the clot; and if used immediately, the serum was inactivated by heating 56°C. for 20 minutes.

NO. OF HOG	RESULT.	REMARKS.
7	+	The blood serum of 54 hogs was tested with antigen No. 2. Of this number only two gave a positive reaction - hyperimmunes Nos. 7 and 294. Hog No. 7 was the hog used in titrating the antigen.
294	+	
601	—	
54	—	
623	—	
21	—	
18	—	
701	—	At the time antigen No. 2 was used on this large group of hogs it was probably not very active as it had been prepared six months previously and the reaction with serum No. 7 and No. 294 was weak. Moreover, a freshly prepared antigen No. 3, which was also titrated against serum No. 7 showed the serum to be strongly positive, as was also serum of hog No. 294. In addition to this the serum from four other hogs, of the opposite column, that reacted negatively to antigen No. 2 showed a positive reaction to the fresh antigen No. 3 - giving further proof of the weakening of antigen No. 2. (See Table No. X..)
19	—	
25	—	
22	—	
26	—	
690	—	
20	—	
23	—	
24	—	
47	—	
48	—	
46	—	
45	—	
50	—	
56	—	
52	—	
60	—	
58	—	
703	—	
59	—	
514	—	40 of the hogs in opposite

NO. OF HOG.	RESULT.	REMARKS.
511	—	column that reacted negatively to the weaker antigen No. 2, were not checked against the stronger antigen No. 3.(The possibility that the same percentage of positive reactions (33 1/3 %) would have been shown, had the entire lot of hogs in the opposite column been tested with the stronger antigen). Moreover the negative reactions shown by some of the animals of opposite column may be due to the fact that they had not been recently hyper-immunized or that the hyper-immunizing blood used was of low virulence. These are factors we have not checked up, on this group of hogs. But Table No.IX. showing positive reactions on a recently hyper-immunized group of hogs has a bearing on this matter.
510	—	
504	—	
512	—	
503	—	
506	—	
505	—	
320 S.	—	
77 S.	—	
96 S.	—	
75 S.	—	
299	—	
501	—	
342	—	
318	—	
274	—	
298	—	
292	—	
324	—	
273	—	
323	—	
272	—	
278	—	
293	—	
264	—	
275	—	
	—	Hog numbers marked S. are ones that were slaughtered at the time the sample of blood was taken.

TABLE NO. IX.

TESTS OF HOG SERUM FROM HYPERIMMUNES,

WITH ANTIGEN NO. 3.

NO. OF HOG.	RESULT.
516	+
408	+
387	+
501	+
273	+
510	+
471	+
488	+
294	+
474	+
465	+
506	+
509	+
382	+
412	+
417	+
386	+
414	+
384	+
413	+
385	+
383	+
563	+
389	+
416	+
564	+
537	+
530	+
545	+
536	+
533	+
524	+
518	+
294	+
7	+
279	—
547	—
552	—
0	—
555	—
550	—
556	—
326	—
74	—

REMARKS.

Antigen No. 3 was used in testing the serum of 67 hyperimmunes; 35 of these gave a positive reaction, and 32 were negative. The higher percentage of positive reactions in this group as compared with the preceding group is in part probably due to the more active antigen used - whether this group was more highly immunized than the preceding group is a matter to be determined..

The important fact developed is that a positive Complement Fixation reaction did occur with the serum of a considerable number of hyperimmunized hogs, when an antigen extract from the spleen pulp of a pig suffering from an acute haemorrhagic type of cholera was used.) A considerable number of the hogs that showed the positive reaction and negative reaction have been retained for further study.

Hog numbers marked S. are ones that were slaughtered at the time the sample of blood was taken.

#3 antg.

NO. OF HOG.	RESULT.	REMARKS.
551	—	
539	—	
513	—	
515	—	
545	—	
542	—	
381	—	
502	—	
548	—	
✓ 504	—	also — #2 antg.
✓ 503	—	" — #2 "
✓ 512	—	also — #2 antg.
✓ 514	—	" — #2 "
464	—	
463	—	
461	—	
472	—	
295 S.	—	
✓ 299 S.	—	also — #2 antg.
279 S.	—	
✓ 292 S.	—	also #2 antg.
✓ 318 S.	—	" #2
✓ 342 S.	—	" #2

TABLE NO. X.

GROUP OF CHOLERA IMMUNES TESTED WITH

ANTIGEN NO. 2 AND NO. 3.

NO. HOG.		ANTIGEN NO. 2	ANTIGEN NO. 3
a.	7	++	++
	294	++	++
b.	501	—	+
	506	—	+
	510	—	+
	273	—	+
c.	504	—	—
	512	—	—
	503	—	—
	299	—	—
	342	—	—
	318	—	—
	292	—	—
	514	—	—

The hogs given in the above table is a regrouping from Tables VIII and IX. of individuals that were tested with both antigens Nos. 2 and 3.

As shown in the previous tables the indications were that antigen No. 2 was the weaker, and this is further borne out by this direct comparison. As already mentioned antigen No. 2 was prepared six months previous to the preparation of antigen No. 3. It will be observed that all the positive reacting serums for antigen No. 2 were also positive for antigen No. 3. And that four of the samples which were negative to antigen No. 2 were

positive to No. 3, while 8 serums were negative to both. The full interpretation of these results remains to be determined. Fuller light will be thrown on the matter by a study of the history of each hog that supplies the serum samples - the number of times injected with virus, the degree of virulence and quantity of infected blood used time intervening between the hyperimmunizing and the drawing of samples for test - differences in samples from first, second and third bleedings.

TABLE NO.XI.

COMPLEMENT FIXATION TEST ON SERUM

OF SICK PIGS, AFFECTED WITH CHOLERA.

DATE.	NO. OF PIG	WEIGHT.	RESULT.
Oct. 14, 1914.	1	45	—
" "	2	45	—
" "	3	45	—
" "	4	50	—
Oct. 17, 1914	5	75	—

The serum samples from pigs Nos. 1 to 4 were drawn from the tail on the seventh day following inoculation with 2 c.c. of hog cholera virus. Pig No. 5 was suffering from natural infection and had a temperature of 105°F. Antigen Extract No. 2 was used in the test of the serum samples. This antigen had been prepared three weeks previously. A negative reaction was given by all samples. The antigen checked positive with serum No. 7.

Antigen No. 3 has not been tried on the serum of sick hogs.

RELATION OF B. SUIPESTIFER TO THE COMPLEMENT FIXATION TEST.

The relation of B. Suipestifer to the positive reactions obtained is a matter of interest. Since that microorganism is often associated with the filterable virus of hog cholera.

Preliminary tests with an antigen prepared from a pure culture of B. Suipestifer gave no deviation of the Complement with serum samples obtained from hogs which gave a positive reaction to antigen extract No. 3 - this seems to indicate that the reactions shown in Table No. IX. are not due to immune bodies against B. Suipestifer.

Two hogs inoculated with cultures of B. Suipestifer had not at the end of seven days produced immune bodies in sufficient quantity to deviate the complement when used either with B. Suipestifer antigen (or the spleen extract antigen No. 3). Moreover the spleen, from hog No. 5, which was used in preparing antigen No. 3, failed to yield cultures of B. Suipestifer.

VARIATIONS AND LOSS OF ANTIBODY CONTENT IN SWINE HYPERIMMUNED TO HOG CHOLERA.

An observation of interest relating to the apparent loss of the cholera immune bodies, in hyperimmunized hogs,- so far as they could be detected by the antigen used - was made during the course of these experiments. And this came from what was at the time thought to be an unfortunate accident. The Titration hog No. 7, the only hog which up to this time had reacted strongly to antigen No. 2 had a leg strained at the third hyperimmunization, and was unable to walk for nearly four months; but by careful nursing recovered and was again used to supply serum for the investigation. The interesting point in the use of this serum was the fact that, after his illness, his serum when tested with the same antigen which, when used before his illness, gave a distinct deviation, now showed no deviation of the complement. Hog No. 7 was then treated with 2 litres of virus, intraperitoneally; and at the end of seven days a sample of blood was taken from his tail for examination. This serum when tested showed a distinct deviation. The reaction was not as strong however as it was six months before, and just

previous to the injury mentioned. This might be due to two causes - first to a lower antibody content of the serum following this re-hypering, as compared with the samples tested six months before, - due to possible differences in the virus used or the method of hyper-immunization - the previous hyperimmunization was by intravenous injection, while the latter was by intra-peritoneal injection. The more probable cause however of the weaker reaction was a loss of strength in the antigen used. It was six months old. Later work with this antigen (No. 2) in comparison with a fresh antigen (No. 3) on the serum of this and other positive reacting hogs as shown in preceding tables justify this interpretation. Another point is that the presence in the circulating blood of a free detectible antibody, (by the antigen used), was not necessary for the maintenance of immunity against hog cholera, of the hog that had been actively hyper-immunized six months before; otherwise the animal would have contracted the disease in a virulent form from the injection of the large quantity of virus intraperitoneally. In this connection may be mentioned the fact that in the practical production of anti-hog-cholera serum, it is necessary to re-hyperimmunize the supply animals at intervals, because of the diminished potency of the serum

in the supply animal from lapse of time and other causes, as is shown by the use of the anti-hog-cholera serum in preventive field work.

The value of an accurate and delicate test to determine the presence and approximate quantity of the hog cholera antibodies in the blood of the supply animals before drawing a large supply for field use is apparent.

RECORD OF CHOLERA PIG USED
IN
EXPERIMENT.

No. 1 Temperature when killed 107.4°F.

Weight 75 lbs.

POST MORTEM LESIONS:

Skin:-- Few hemorrhagic spots.

Lymph glands:

Sub Maxillary:-- Congested.

Cervical:-- Congested.

Bronchial:-- Congested.

Portal glands:-- Enlarged, congested and haemorrhagic

Mesenteric:-- Greatly enlarged and congested.

Inguinal glands:-- Enlarged.

Heart:-- Normal.

Lungs:-- Very slightly pneumonic.

Spleen:-- Greatly enlarged.

Kidneys:-- Slightly petechiated.

Intestines:-- Normal.

Remarks:-- This pig was injected with 2 c.c. of virus and was killed at the end of 10 days, having been sick three days.

RECORD OF CHOLERA PIG USED
IN
EXPERIMENT.

No. 2 Temperature when killed 107.4° F.

Weight 80 lbs.

POST MORTEM LESIONS:

Skin:-- Few hemorrhagic spots.

Lymph glands:

Sub Maxillary:-- Slightly congested.

Cervical:-- Slightly congested.

Bronchial:-- Slightly congested.

Portal glands:-- Greatly congested. Hemorrhagic.

Mesenteric:-- Slightly congested.

Inguinal glands:-- Enlarged, slightly congested.

Heart:- Normal.

Lungs:-- Slightly pneumonic.

Spleen:-- Slightly enlarged.

Kidneys:-- Petechiated.

Intestines:-- Normal.

Remarks:-- This pig was injected with 2 c.c. of virus and was killed at the end of 10 days, having been sick three days.

RECORD OF CHOLERA PIG USED
IN
EXPERIMENT.

No. 3 Temperature when killed 107.6°F.

Weight 75 lbs.

POST MORTEM LESIONS:

Skin:-- Hemorrhagic splotches.

Lymph glands:

Sub Maxillary:-- Congested and haemorrhagic.

Cervical:-- Hyperaemic.

Bronchial:-- Hyperaemic.

Portal glands:- Congested and haemorrhagic.

Mesenteric:-- Slightly congested, greatly enlarged.

Inguinal glands:-- Hyperaemic and enlarged.

Heart:-- Normal.

Lungs:-- Small congested areas.

Spleen:-- Enlarged.

Kidneys:-- Petechiated.

Intestines:-- Normal.

Remarks:-- This pig was injected with 2 c.c. of virus and was killed at the end of 10 days, having been sick three days.

Note--- Mucous membrane of bladder congested.

RECORD OF CHOLERA PIG USED
IN
EXPERIMENT.

No. 4 Temperature when killed 108.4° F.

Weight 75 lbs.

POST MORTEM LESIONS:

Skin:-- Few hemorrhagic spots.

Lymph glands:

Sub Maxillary:-- Enlarged and slightly congested.

Cervical:-- Enlarged and slightly congested.

Bronchial:-- Enlarged and slightly congested.

Portal glands:-- Congested slightly.

Mesenteric:-- Enlarged.

Inguinal glands:-- Enlarged.

Heart:-- Normal.

Lungs:-- Slightly pneumonic.

Spleen:-- Enlarged.

Kidneys:-- Petechiated.

Intestines:-- Normal.

Remarks:-- This pig was injected with 2 c.c. of virus and was killed at the end of 10 days, having been sick three days.

RECORD OF CHOLERA PIG USED
IN
EXPERIMENT.

No. 5 Temperature when killed (Died from cholera)

Weight 30 lbs.

POST MORTEM LESIONS:

Skin:-- Normal.

Lymph glands:--

Sub Maxillary:-- Highly congested and hemorrhagic.

Cervical:-- Highly congested and hemorrhagic.

Bronchial:-- Highly congested and hemorrhagic.

Portal glands:-- Enlarged and congested. Hemorrhagic.

Mesenteric:-- Enlarged and hemorrhagic.

Inguinal glands:-- Greatly enlarged and hemorrhagic.

Heart:-- Normal.

Lungs:-- Numerous petechiae and with areas of ecchymoses.

Spleen:-- Greatly enlarged.

Kidneys:-- Highly petechiated.

Intestines:-- Normal.

Remarks:-- This pig had been dead a few hours when post mortem was made and spleen and kidneys taken for extraction.

Memo: pigs 6 & 7 from Oaine farm.

RECORD OF CHOLERA PIG USED
IN
EXPERIMENT.

No. 6 Temperature when killed 104°F.

Weight 30 lbs.

POST MORTEM LESIONS:

Skin:-- Normal.

Lymph glands:--

Sub Maxillary:-- Enlarged, highly congested, hemorrhagic.

Cervical:-- Enlarged, congested and hemorrhagic.

Bronchial:-- Enlarged, congested and hemorrhagic.

Portal glands:-- Enlarged, congested and hemorrhagic.

Mesenteric:-- Enlarged, congested, hemorrhagic.

Inguinal glands:-- Enlarged, congested, hemorrhagic.

Heart:-- Normal.

Lungs:-- Petechiated.

Spleen:-- Greatly enlarged.

Kidneys:-- Numerous petechiae.

Intestines:-- Normal.

Remarks:-- This pig was naturally infected and was killed by etherizing when showing most acute symptoms of cholera.

meins.
< from Dane farm as pig no 5 >

RECORD OF CHOLERA PIG USED
IN
EXPERIMENT.

No. 7 Temperature when killed 101°F.

Weight 30 lbs.

POST MORTEM LESIONS:

Skin:-- Normal.

Lymph glands:

Sub Maxillary:-- Enlarged, highly congested, hemorrhagic.

Cervical:-- Enlarged, highly congested, hemorrhagic.

Bronchial:-- Enlarged, highly congested, hemorrhagic.

Portal glands:-- Enlarged, highly congested, hemorrhagic.

Mesenteric:-- Enlarged, highly congested, hemorrhagic.

Inguinal glands:-- Enlarged, highly congested, hemorrhagic.

Heart:-- Normal.

Lungs:-- Petechiated.

Spleen:-- Greatly enlarged.

Kidneys:-- Numerous petechiae.

Intestines:-- Normal.

Remarks:-- This pig was naturally infected and was killed by etherizing when showing most acute symptoms of cholera.

< From farmer Davis, Pig No 506 >

RECORD OF CHOLERA PIG USED
IN
EXPERIMENT.

No. 8 Temperature when killed (Pig died)

Weight 10 lbs.

POST MORTEM LESIONS:

Skin:-- Severely hemorrhagic.

Lymph glands:

Sub Maxillary:-- Highly congested and hemorrhagic.

Cervical:-- Highly congested and hemorrhagic.

Bronchial:--Highly congested and hemorrhagic.

Portal glands:-- Highly congested and hemorrhagic.

Mesenteric:-- Highly congested and hemorrhagic.

Inguinal glands:-- Highly congested and hemorrhagic.

Heart:--Normal.

Lungs:-- Highly petechiated.

Spleen:-- Enlarged.

Kidneys:-- Highly congested and petechiated.

Intestines:-- Normal.

Remarks:-- The liver of this pig seemed to be greatly enlarged. It was used to make an extract -- See under "Method of Preparation of Antigen Extract."

GENERAL OBSERVATIONS.

1. A complement deviating antigen for anti-hog-cholera serum does not exist apparently in the spleens of all pigs suffering from hog cholera.

2. The blood serum from pigs affected with acute cholera did not give a deviating antigen.

3. The two antigens used differed considerably in strength.

4. More than fifty percent of all hyperimmunes tested reacted positively to antigen No. 3 - spleen extract from pig No. 5.

5. Hog serum was highly hemolytic for the red cells of Horse No. 1, of goat No. 1, and cows Nos. 1 and 2, but not for cow No. 3.

6. There is no difference in the hemolytic power of serum from normal, hyperimmunes, and cholera infected hogs.

7. All cholera infected pigs' serum tested reacted negatively.

8. Whether the immune body in the more than 50% of hogs tested with antigen No. 3 is the specific protective body that prevents hog cholera is a matter for more extended research.

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UNIVERSITY OF MISSOURI
COLUMBIA

PREVENTIVE MEDICINE

May 26, 1915.

Dean Walter Miller,
211 Academic Hall,

Dear Sir,--

It gives me pleasure to approve the enclosed thesis submitted by Mr. A. J. Durant for the degree of Master of Arts. In my opinion it meets fully our standards and represents a large amount of painstaking work on a difficult subject which he has presented very clearly.

Very truly yours,

D. H. Dalby

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