Issued April 7, 1911.

U. S. DEPARTMENT OF AGRICULTURE,
BUREAU OF ANIMAL INDUSTRY.—BULLETIN 136.

A. D. MELVIN, CHIEF OF BUREAU.

THE DIAGNOSIS OF GLANDERS BY
COMPLEMENT FIXATION.

BY

JOHN R. MOHLER, V. M. D.,
Chief of the Pathological Division,

AND

ADOLPH EICHHORN, D. V. S.,
Bacteriologist, Pathological Division.

[Reprint, October, 1911, with slight revision.]

WASHINGTON:
GOVERNMENT PRINTING OFFICE.
1911.

Class SF796

Book M6

7
Issued April 7, 1911.

U. S. DEPARTMENT OF AGRICULTURE,
BUREAU OF ANIMAL INDUSTRY.—BULLETIN 136.

A. D. MELVIN, CHIEF OF BUREAU.

THE DIAGNOSIS OF GLANDERS BY
COMPLEMENT FIXATION.

BY

JOHN R. MOHLER, V. M. D.,
Chief of the Pathological Division,

AND

ADOLPH EICHHORN, D. V. S.,
Bacteriologist, Pathological Division.

[Reprint, October, 1911, with slight revision.]

WASHINGTON:
GOVERNMENT PRINTING OFFICE.
1911.

Copy 2

SF796
M6

THE BUREAU OF ANIMAL INDUSTRY.

Chief: A. D. MELVIN.

Assistant Chief: A. M. FARRINGTON.

Chief Clerk: CHARLES C. CARROLL.

Animal Husbandry Division: GEORGE M. ROMMEL, chief.

Biochemic Division: M. DORSET, chief.

Dairy Division: B. H. RAWL, chief.

Inspection Division: RICE P. STEDDOM, chief; R. A. RAMSAY, MORRIS WOODEN,
and ALBERT E. BEHNKE, associate chiefs.

Pathological Division: JOHN R. MOHLER, chief.

Quarantine Division: RICHARD W. HICKMAN, chief.

Zoological Division: B. H. RANSOM, chief.

Experiment Station: E. C. SCHROEDER, superintendent.

Editor: JAMES M. PICKENS.

U. S. D.
NOV 6 1911

LETTER OF TRANSMITTAL.

U. S. DEPARTMENT OF AGRICULTURE,
BUREAU OF ANIMAL INDUSTRY,
Washington, D. C., March 20, 1911.

SIR: I have the honor to transmit herewith a paper entitled "The Diagnosis of Glanders by Complement Fixation," by Drs. John R. Mohler and Adolph Eichhorn, of the Pathological Division of this bureau.

Since the discovery of the glanders bacillus in 1883 many efforts have been made to find a reliable method of making an early diagnosis of the disease. The mallein test and later the agglutination test have been and are at present in general use, but neither of these is sufficiently reliable to be entirely satisfactory. Schütz and Schubert, German investigators, recently called attention to the value of complement fixation as affording a more reliable method of diagnosing glanders, and within the past year this method has been carefully studied and tested in the Pathological Division of this bureau.

It will be seen from the details given in the accompanying paper that the authors have found the complement fixation test to be highly reliable as a diagnostic agent for glanders, and they present a thorough exposition of the method resulting from their searching experiments, including practical tests in a recent outbreak of glanders at Washington, D. C.

In view of the great economic and scientific importance of the subject, and as no work upon this new method has so far been published in the United States, I recommend the immediate publication of the paper in the bulletin series of this bureau, in order that the value of the method and the technique necessary for its application may be made more fully available in this country.

Respectfully,

A. D. MELVIN,
Chief of Bureau.

Hon. JAMES WILSON,
Secretary of Agriculture.

CONTENTS.

	Page.
Introduction	5
Hemolysis	7
Method of obtaining hemolytic amboceptor (rabbit serum)	9
Titration of hemolytic rabbit serum	12
Method of obtaining complement (guinea-pig serum)	14
Titration of complement	14
Specific complement fixation (deviation)	16
Method of obtaining serum of animals to be tested	18
Inactivating the serum	19
Preparation of the antigen (glanders bacilli extract)	20
Titration of the extract	21
The complement-fixation test	23
Application of the test	24
Controls	25
Interpreting results of tests	27
Controlling glanders in an infected stable	29
Results of practical tests with complement fixation	29

ILLUSTRATIONS.

	Page.
PLATE I. Diagrammatic representation of complement fixation	8
II. Titration of hemolytic amboceptor (rabbit serum)	12
III. Titration of complement (guinea-pig serum)	16
IV. Titration of antigen (glanders bacilli extract)	20
V. Final test showing positive reaction to glanders	26

THE DIAGNOSIS OF GLANDERS BY COMPLEMENT FIXATION.

INTRODUCTION.

The early diagnosis of glanders constitutes one of the most important and difficult tasks which confronts the veterinarian engaged in sanitary work. This of course does not apply to the clinical cases of glanders, as in such cases the diagnosis is usually made without much difficulty from the characteristic symptoms and lesions present. In those instances, however, where there are no positive indications of the disease, it is impossible to establish a diagnosis by physical examination, and only through the aid of some special diagnostic method or test can there be any hope of determining the presence or absence of the disease. Horses affected with occult or latent glanders, and in which the disease is not suspected, are undoubtedly great factors in the propagation of the infection. Indeed, there are many glandered horses which do not show positive symptoms until the later stages of the disease.

Since the discovery of the glanders bacillus in 1883 by Loeffler and Schütz the diagnosis of glanders has been the subject of numerous investigations, and as a result great progress has been made in its determination. After the isolation of the infective agent of the disease the diagnosis was confined to the demonstration and cultivation of the organism, or to the reproduction of the disease by inoculations of exudates or parts of diseased organs from affected horses into susceptible animals.

The first important step toward determining obscure and latent cases of glanders was made by the discovery of mallein. With the aid of this biological product of the *Bacillus mallei* a large proportion of latent and occult cases of glanders can be diagnosed, particularly when such tests are made by efficient and experienced veterinarians. There are, however, a considerable number of glanderous animals in which the mallein fails to give a typical reaction, and, on the contrary, a reaction may follow the injection of mallein in the absence of glanders. Thus mallein is not an entirely reliable diagnostic agent for determining glanders, nor has it ever been considered

as efficacious in the detection of this disease as tuberculin for the diagnosis of tuberculosis.

With the application of the agglutination test for glanders it appeared that a more satisfactory method had been found for the diagnosis of all types of infection with this disease. It was first suggested by McFadyean in 1896, after this investigator had observed the value of Widal's typhoid-fever agglutination test, but was not generally adopted until the method was perfected by Schütz and Meissner, whose interesting results were published in 1905. This test has since been extensively employed in practically every country where glanders exists, and therefore ample opportunity has been furnished for drawing conclusions relative to its diagnostic value.

While there is no doubt that the agglutination test is of great value in all cases of recent infection, the blood in such cases possessing a very high agglutination power (1 to 1,000 and higher), nevertheless extensive experience has proved that horses affected with chronic glanders give occasionally a very low agglutination value, which in some cases is even lower than that of normal blood serum (1 to 400 or even lower). From this condition it appears evident that in certain cases of chronic glanders the disease can be determined only by repeated tests, and a diagnosis in such cases is only possible from the fluctuation of the agglutination value—either an increase or a decrease—as it is a well-known fact that this value remains stationary in normal horses.

Besides this difficulty, there should also be taken into consideration the fact that the blood of normal horses sometimes shows a high agglutination value (1 to 800 and higher), and that changes in the agglutination power have been observed even in animals free of glanders. Furthermore, repeated agglutination tests require considerable time, as at least two weeks should elapse between two tests. Therefore the agglutination test alone does not constitute an entirely satisfactory diagnostic method for glanders. However, as its great value has been proved beyond doubt in the early cases of infection, it may well be utilized as an adjunct to any other test which may be applied in connection with the diagnosis of suspected cases of the disease.

Hutyra compared the agglutination test with the mallein test from the tables included in the works of Schütz and Miessner and of Nevermann, and came to the conclusion that the application of the agglutination test alone has not decreased the number of faulty diagnoses. He believes that the principal difference in the results lies in the fact that a large number of horses which were classified as only suspicious by the mallein test are considered as actually infected by the agglutination test.

In further efforts to find a method by which an early diagnosis of glanders could be made, various investigators directed their attention to the precipitation reaction. This is based upon the fact that when blood serum comes in contact with a concentrated extract of glanders bacilli the precipitins or receptors, which are formed in the blood of infected animals from the time the infection first occurs, are bound to the bodies in the bacillary extract, producing a precipitation which is manifested by cloudiness at the point of contact of the two fluids. This method of diagnosing glanders has recently been recommended by Pfeiler¹ in Germany and by Konew² in Russia, but it has not been applied extensively in practice. This is probably due to the fact that the reading of the reaction is in some cases difficult, due to the indistinct ring which occasionally is formed at the line of contact between the precipitant and the serum.

In 1909 Schütz and Schubert³ published the results of their important work on the application of the method of complement fixation for the diagnosis of glanders. And since their experiments were followed by splendid results, exceeding by far the results obtained from either the mallein or the agglutination test, they recommended that this method of diagnosis in combination with the agglutination test be taken as the official test in Germany. This method, overcoming as it does the disadvantages of the mallein and agglutination tests, constitutes without doubt the most reliable method for the diagnosis of glanders which we have at our command at the present time. The complement-fixation test is, in fact, the most definite method known for determining specific infections and is as nearly perfect as a biological test can be. It has only recently been introduced in veterinary science and the publications concerning it are at present limited exclusively to foreign periodicals. The principle of this test is presented in the phenomenon of hemolysis, which was first discovered and studied by Bordet and Gengou, and extended by Ehrlich, Morgenroth, and Sachs.

HEMOLYSIS.

It is a well-known fact that if red blood corpuscles of one animal are introduced into another of a different species the blood of the latter acquires the power to dissolve the blood corpuscles of the

¹ Pfeiler, Willy. Die Ermittlung der Rotzkrankheit durch die Präzipitationsmethode. Archiv für Wissenschaftliche und Praktische Tierheilkunde. Band 35, Heft 4/5, pp. 323-337. June 24, 1909.

² Konew, D. Präzipitationsreaktion als diagnostische Methode beim Rotz. Vorläufige Mitteilung. Centralblatt für Bakteriologie. Abt. 1, Orig., Band 55, Heft 3, pp. 251-253. July 9, 1910.

³ Schütz and Schubert. Die Ermittlung der Rotzkrankheit mit Hilfe der Komplementablenkungsmethode. Archiv für Wissenschaftliche und Praktische Tierheilkunde. Band 35, Heft 1/2, pp. 44-83. 1909.

former when mixed with them in a reagent glass. This reaction is termed hemolysis, which means the dissolution of blood corpuscles, thereby setting the hemoglobin free in the medium in which the corpuscles are suspended.

To illustrate this phenomenon, if a rabbit is injected intraperitoneally, intravenously, or subcutaneously with washed red blood corpuscles of a sheep, the blood of the rabbit will develop antibodies which possess a dissolving action for the sheep blood corpuscles; that is, the rabbit blood will contain specific hemolysins.

The acquired hemolytic property of the blood depends on two substances. One of these is present in the blood of every animal, and is known as the complement. It is thermo-labile, which means that it is rendered inactive after the blood or serum has been heated to 56° C. for half an hour. The other body, which is formed as a result of the injection of blood corpuscles, is thermo-stable; that is, it resists heating even higher than 56° C., and is known as immune body, fixative, sensitizer, or hemolytic amboceptor. The name amboceptor is derived from the fact that it has an affinity on the one hand for the blood corpuscles of the species of animal with which the animal has been injected, and on the other for the complement, this union taking place only after the first-mentioned affinity has been satisfied.

These two substances, together with the corpuscles to be dissolved, comprise the hemolytic system, and their combination leads to hemolysis. (See Pl. I, A.) This means that an opaque suspension of blood corpuscles is rendered semitransparent or "laked." The hemolysis, strictly speaking, does not represent a complete solution, but only an action of the hemolysin on the stroma of the erythrocytes, which permits the escape of the hemoglobin of the red blood corpuscles.

The injection of blood corpuscles of one animal into another of a different species gives rise to the development of antibodies which confer upon the blood serum the hemolytic action. This phenomenon is somewhat similar to the production of receptors in the formation of antitoxins which are thrown off, but these receptors alone are not able to dissolve the red blood corpuscles, requiring also the presence of a ferment. This ferment, however, is a constant constituent of the blood and is known as the complement.

That both of these substances are constantly present in the hemolytic serum can be demonstrated in the following manner: If the hemolytic serum is heated to 56° C. for half an hour, thereby destroying the complement, this serum will no longer possess a hemolytic action; that is, it will no longer dissolve red blood corpuscles. This heating of the serum is known as inactivation. On the other hand, if to such inactivated serum there be added fresh untreated

DIAGRAMMATIC REPRESENTATION OF COMPLEMENT FIXATION.

- A, Hemolytic system.
- B, Bacteriolytic system.
- C, Negative reaction with normal horse serum.
- D, Positive reaction with glandered horse serum.

serum, which in itself has no hemolytic properties, hemolysis will result. Thus by the addition of this fresh serum a reactivation is accomplished. This is explained by the fact that through the heating of the serum one of the substances necessary for the hemolysis has been destroyed, which is the complement. After the complement has been destroyed by heating it can be replaced by the addition of any normal serum, because it is known that the complement is present in all blood. However, the guinea-pig serum appears to be the most satisfactory in the application of hemolysis, inasmuch as it is very rich in complement and only a very small quantity is required to be added to the inactivated hemolytic serum in order to produce hemolysis.

Accordingly, the substances necessary for hemolysis are (1) the hemolytic amboceptor, which is the serum of a rabbit that has been injected with washed sheep blood corpuscles, (2) the complement in the form of normal guinea-pig serum, and (3) washed red blood corpuscles of a sheep. In the preparation of these different substances it is necessary to fix standards of practical constancy by proceeding along definite lines in the following manner:

METHOD OF OBTAINING THE HEMOLYTIC AMBOCEPTOR (RABBIT SERUM).

Strong, vigorous rabbits are selected, and they are injected intraperitoneally with a suspension of washed red blood corpuscles from a sheep. Three injections are made at intervals of seven days with 7 c. c., 10 c. c., and 12 c. c. of these blood corpuscles suspended in like quantities of physiological salt solution.¹

The sheep blood is obtained by bleeding a vigorous sheep from the jugular. The side of the neck is clipped and shaved, and the part over the jugular disinfected with 75 per cent alcohol. Then a sterilized small-calibered trocar is inserted into the jugular, and the blood is collected in a sterile bottle containing a few glass beads. After the desired quantity of blood is obtained it is shaken for 10 minutes in order to defibrinate it. After defibrination, it is filtered through a double layer of sterile gauze into the glass tube in which the washing is to take place. The glass tube containing the blood is then filled with salt solution and placed in a centrifuge which has a speed of 2,500 to 3,000 revolutions per minute. After the red blood corpuscles are thrown down the clear fluid above the corpuscles is pipetted off. Then the blood corpuscles are again thoroughly mixed with salt solution in the proportion of 1 to 9, and the centrifugalization is repeated. This washing should be carried out three or even four times in order to eliminate all the serum adhering to the red

¹ Unless otherwise stated, the term "salt solution" in this bulletin refers to an 0.85 per cent solution of sodium chlorid.

blood corpuscles. Such washed blood corpuscles can then be used for the injection of rabbits in the preparation of hemolytic amboceptors, as well as for the test proper.

The washing of the sheep blood corpuscles must be thoroughly carried out, inasmuch as the presence of even traces of serum adhering to the corpuscles may cause difficulty in obtaining satisfactory results. If rabbits were injected with red blood corpuscles containing a small quantity of serum, the rabbits would develop, not only antibodies, or immune bodies, but also coagulins and anticomplements, and the presence of these substances would give rise to difficulties in demonstrating the presence or absence of a complete hemolysis. Furthermore, if blood corpuscles containing even traces of serum were used in the tests it might produce a fixation of the complement and thereby give rise to errors. Such errors would occur particularly if the hemolytic action of the rabbit serum was not very high.

The washed blood corpuscles should be used for the injection of the rabbit on the day the blood is drawn. For testing purposes, however, it will keep for two or three days in the ice chest.

The rabbits to be injected are shaved on the posterior part of the abdomen, and the skin is disinfected with 75 per cent alcohol. They are then held with the head down by an assistant in order to prevent the puncturing of the intestines. The blood corpuscles to be injected are mixed with an equal quantity of salt solution and heated in a water bath to body temperature. Intravenous injections have been recommended by some investigators, but it was found in our work that intraperitoneal injections gave very satisfactory results, and furthermore, there is very little danger of losing rabbits from various complications by this method.

After three injections with the quantities and at the intervals stated above, a small amount of blood is taken from the rabbit on the fifth or sixth day after the last injection. This blood is then titrated in order to determine whether its hemolytic action is of sufficient strength for future work. If the blood serum is found to be of a sufficiently high titre, the rabbit may be either bled to death, or, what is far more satisfactory, about 15 c. c. of blood may be drawn from the veins of the ear. By the latter method the rabbit may be used continuously for the production of hemolytic serum. If it is desired to obtain the blood by bleeding the rabbit to death, the animal is anesthetized by a mixture of chloroform and ether, the hair on the neck is shaved, the skin is disinfected, and an incision made on one side of the neck in order to sever both the jugular vein and the carotid artery. Should the flow of blood cease on this side, the other side may also be cut. The blood is collected in centrifuge

tubes, and after the bleeding has been completed centrifugalization of the blood is accomplished and the supernatant serum drawn off with a pipette.

Bleeding of the rabbit through the veins of the ear is best accomplished in the following way: After washing the ear and closely clipping the hair over the veins on the outside of the ear, the skin is disinfected with 75 per cent alcohol. Then a pledget of cotton is soaked in hot water (about 45° or 50° C.) and wound around the base of the ear in order to produce a hyperemia of the blood vessels. When a sufficient dilation of the vessels is observed, the middle and posterior auricular veins are severed, and the blood is then collected in centrifuge tubes. If the blood ceases to flow, the cotton should be removed from the base of the ear, and after placing it in hot water it is again applied to the ear and the hyperemia is thereby reestablished. In case the coagulation of the blood has prevented its flow at the place where the veins were severed, the wound may be scraped with a knife, and usually the blood will commence to flow freely again. The collected blood is then treated in the same manner as described above—that is, centrifuged—and the supernatant serum pipetted off.

Should this hemolytic rabbit serum be used before it is 3 days old, it must be inactivated by heating to 56° C. for one-half hour. After this time the hemolytic rabbit serum is preserved with one-half of 1 per cent of carbolic acid—that is, to each 9 c. c. of serum 1 c. c. of a 5 per cent carbolic-acid solution is added. Serum preserved in this way may be kept for two or three months. However, it should be retitrated every two or three weeks, as occasionally the titre of the serum drops. Such carbolized hemolytic serum does not require inactivation.

When preparing hemolytic serum it is best to start with several rabbits, as occasionally one may die as a result of anaphylaxis, and, again, some rabbits are not adapted for the production of hemolysins.

The titre of the hemolytic serum from a rabbit does not remain stationary, but for two or three weeks after the last injection it gradually lowers until it reaches that of a normal animal. If, however, such a rabbit is reinjected with washed corpuscles after several weeks, the hemolysins will again appear after a short time. Hemolysins are kept more or less in reserve in the cells of such an animal. The renewed injection acts as a stimulant, and these bodies are quickly thrown off into the blood, while in a normal animal they form slowly, first being formed in the cells. Thus, the advisability of keeping rabbits which have been bled from the veins of the ear for further production of hemolytic amboceptors can readily be seen.

TITRATION OF HEMOLYTIC RABBIT SERUM.

The hemolytic rabbit serum is titrated in order to establish the smallest quantity of serum that will produce hemolysis in the presence of a certain quantity of complement and the suspension of washed blood corpuscles of a sheep. The amount of hemolytic serum that will produce complete hemolysis in two hours at 37° C. is an amboceptor unit.

For the preliminary work of titration 10 test tubes are taken, and dilutions of the hemolytic serum are made with salt solution in proportions of 1 to 100, 200, 400, 500, etc., up to 4,000. These dilutions are made from basic dilutions of 1 to 100 and 1 to 1,000 as may be seen from Table 1.

TABLE 1.—*Titration of rabbit serum (hemolytic amboceptor).*

Tube.	NaCl solution. ¹	Amboceptor.	Complement. ²	Blood corpuscles. ³	Remarks.
	c. c.	1 c. c. of—	c. c.	c. c.	
1	2.5	1: 100.....	0.5	1	
2	2.5	1: 200.....	.5	1	
3	2.5	1: 400.....	.5	1	
4	2.5	1: 500.....	.5	1	
5	2.5	1: 600.....	.5	1	
6	2.5	1: 800.....	.5	1	
7	2.5	1:1,000.....	.5	1	
8	2.5	1:1,500.....	.5	1	
9	2.5	1:2,000.....	.5	1	
10	2.5	1:4,000.....	.5	1	
11	3.5		.5	1	Complement control (no hemolysis should occur).
12	3.0	1: 100.....		1	Amboceptor control (no hemolysis should occur).
13	4.0			1	Salt-solution control (no hemolysis should occur).

¹ 0.85 per cent NaCl solution.

² 0.5 c. c. of a 10 per cent solution of the complement.

³ 5 per cent suspension of sheep-blood corpuscles washed in salt solution.

From each batch of serum taken from the rabbit a basic dilution is made in the proportion of 1 to 100, 200, 400, etc., up to 4,000. From these basic dilutions the third column in Table 1 is made, as indicated below:

A dilution of—

200 is made by adding 1 c. c. of 1 to	100 dilution to 1 c. c. NaCl solution.
400 is made by adding 1 c. c. of 1 to	100 dilution to 3 c. c. NaCl solution.
500 is made by adding 1 c. c. of 1 to	100 dilution to 4 c. c. NaCl solution.
600 is made by adding 1 c. c. of 1 to	100 dilution to 5 c. c. NaCl solution.
800 is made by adding 1 c. c. of 1 to	100 dilution to 7 c. c. NaCl solution.
1,000 is made by adding 1 c. c. of 1 to	100 dilution to 9 c. c. NaCl solution.
1,500 is made by adding 2 c. c. of 1 to	1,000 dilution to 1 c. c. NaCl solution.
2,000 is made by adding 1 c. c. of 1 to	1,000 dilution to 1 c. c. NaCl solution.
4,000 is made by adding 1 c. c. of 1 to	1,000 dilution to 3 c. c. NaCl solution.

It will be observed that the last three are made from the 1 to 1,000 dilution.

TITRATION OF HEMOLYTIC AMBOCEPTOR (RABBIT SERUM).
(Tube 8 is highest titration showing hemolysis, and therefore represents the titre.)

The titration proper is then made in the following manner: Ten additional test tubes are each filled with 2.5 c. c. of salt solution, to which is then added the hemolytic serum (amboceptor) in quantities of 1 c. c. of the different dilutions to each tube. Thus, in the first tube we add 1 c. c. of the dilution of 1 to 100; in the next, 1 c. c. of the dilution of 1 to 200; and in the third, 1 c. c. of the dilution of 1 to 400, etc. Afterwards the complement of the guinea-pig serum is added in quantities of 0.5 c. c. of a 10 per cent solution to each tube, and finally 1 c. c. of a 5 per cent suspension of sheep corpuscles in salt solution is placed in each tube.

Besides these 10 tubes, there are also three control tubes, one to show that the complement alone (without the amboceptor) will not produce hemolysis, the second that the amboceptor alone without the complement will not produce hemolysis, and the third that the salt solution alone will not produce hemolysis. Thus, in the first control tube we add 3.5 c. c. of salt solution, 0.5 c. c. complement, and 1 c. c. suspension of sheep corpuscles. In the second control tube we add 3 c. c. salt solution, 1 c. c. amboceptor of the 1 to 100 dilution, and 1 c. c. suspension of sheep corpuscles. In the third control tube we add 4 c. c. salt solution and 1 c. c. sheep corpuscles. It is advisable to place the tubes for the titration test in the lower row and the control tubes in the upper row of the test-tube rack.

As can be seen from the various quantities added to the tubes, the final volume is always uniformly 5 c. c. Thus, the different amounts of the blood derivatives used are always made up to 5 c. c. by the addition of salt solution.

After adding the substances in the test tubes in the order given, the tubes are well shaken, placed in a rack, and the rack put in an incubator at 37° C. for two hours. Then it is removed from the incubator and the results are read. (See Pl. II.)

The highest dilution in which complete hemolysis has taken place represents the titre of the hemolytic amboceptor. Thus, if complete hemolysis has taken place in the tubes where the dilution used in the rabbit serum was 1 to 2,000, the hemolytic amboceptor of that serum is represented by 2,000. This titre, however, is not used in the glanders test, but rather its double strength, which would be 1 to 1,000.

The titre of the hemolytic amboceptor for use in glanders diagnosis should not be less than 1 to 1,000, and therefore if the rabbit serum should prove of a lower titre it should not be used for this test. A low titre of the hemolytic amboceptor would disturb the results of the test, inasmuch as in such low dilutions too much serum would have to be used, and as the serum contains other substances in addition to amboceptors it would have an influencing effect on the hemolysis.

It is advisable to preserve the carbolized hemolytic amboceptor in small vials containing 1 to 2 c. c. of the rabbit serum, sealing them with paraffin. By this procedure the contents of a vial are used up more quickly and without frequent exposure.

The titration test of the hemolytic amboceptor is given in consecutive order in Table 1, and the method of carrying out the tests relative to the addition of the different substances is likewise given in consecutive order in all the tables. Thus the number of test tubes used is indicated, as well as the various substances and the quantities to be added, as they follow each other. It is our opinion that by this method the description of the tests can be followed without much difficulty, and by keeping these tables in the foreground the tests themselves may be applied readily, even by those who have had but little experience in this work. Of course accuracy in technique is the most important factor in the success of this line of serum diagnosis, and too much emphasis can not be laid upon this fact.

METHOD OF OBTAINING COMPLEMENT (GUINEA-PIG SERUM).

The complement represents the blood serum of a healthy guinea pig, and is obtained by bleeding the animal by severing the carotid and the jugular.

The guinea pig is anesthetized with a mixture of chloroform and ether, and the neck is shaved and then disinfected with a 75 per cent solution of alcohol. The animal is held by an assistant with its head down, while the operator uses his left hand to pull taut the skin over the region of the throat and his right hand to make an incision on one side of the neck by which the carotid and the jugular are severed. The centrifuge tube is immediately held under the opening, in order to collect all the blood. After the flow ceases, the same technique is practiced on the other side of the neck. Care should be taken to avoid the cutting of the trachea.

The blood thus collected, which usually amounts to 10 or 12 c. c., is placed in the ice chest, and after one or two hours the serum separates from the clot. The serum is then drawn off, and the rest is placed in a centrifugal machine, in order to obtain all the serum present in the clot. The guinea-pig serum should always be used fresh, and it is never advisable to use it after the second day, as the complement becomes considerably reduced upon standing.

TITRATION OF COMPLEMENT.

It is desirable to titrate the complement of every guinea pig, as such practice will insure more accurate work and better results in the glanders test. By titration of the complement we aim to estab-

lish the complement unit which is the smallest quantity of complement necessary to produce complete hemolysis in the presence of one amboceptor unit and a suspension of blood corpuscles of sheep. The smallest quantity is then taken as its test value. For the titration of the complement six test tubes are used, in addition to the three for controls. A 10 per cent basic dilution is made of the complement in the first test tube; that is, to 2.7 c. c. of salt solution 0.3 c. c. of complement is added. From this basic dilution the other dilutions are made by consecutively reducing the quantity of complement in the different test tubes. Thus, in the second test tube 0.5 c. c. of the 10 per cent basic dilution is added, in the third 0.4 c. c., in the fourth 0.3, and so on. Of course we add to each of these test tubes sufficient quantities of salt solution to make the required 3 c. c. Thus, into the second test tube we add 2.5 c. c., in the third 2.6 c. c., etc., of salt solution. After the complement has been added in quantities stated above, it is followed by the amboceptor. One cubic centimeter of the hemolytic amboceptor of which the titre has previously been determined is added, and finally 1 c. c. of a 5 per cent emulsion of blood corpuscles. The purpose of the controls is to establish in the first that the complement alone without the amboceptor does not produce hemolysis; in the second, that the amboceptor alone without the complement produces no hemolysis; and in the third, that the salt solution alone does not produce hemolysis. The first control tube contains 3.5 c. c. salt solution, 0.5 c. c. of the 10 per cent basic dilution, and 1 c. c. of suspension of corpuscles; in the second control 3 c. c. of salt solution, 1 c. c. of the amboceptor dilution, and 1 c. c. of suspension of blood corpuscles; in the third control, 4 c. c. of salt solution and 1 c. c. of suspension of blood corpuscles are used.

After shaking all the tubes, the rack is placed in the incubator for two hours, and removed in order to read the results. (See Pl. III.) The highest dilution of complement in the tube in which the hemolysis is complete indicates the titre of the complement. For instance, if hemolysis is complete in the tube where 0.3 c. c. of the 10 per cent basic dilution of the complement has been used, and hemolysis is incomplete in the tube in which 0.2 c. c. of a 10 per cent basic dilution has been used, then the titre of the complement is 0.03 c. c., inasmuch as we have started with a 10 per cent basic dilution. Thus, in the tests in this instance it would be necessary to use a 3 per cent complement solution.

In Table 2, the titration of the complement is given as in Table 1 for the hemolytic amboceptor, and this can be followed without much difficulty. Particular care, however, should be taken to use exact quantities as designated in the table.

TABLE 2.—*Titration of complement.*

Tube.	NaCl solution. ¹	Complement. ²	Amboceptor. ³	Blood corpuscles. ⁴	Remarks.
	c. c.	c. c.	c. c.	c. c.	
1	2.7	0.3	-----	-----	Basic dilution of complement.
2	2.5	.5	1	1	Tubes 2 to 6 are for establishing the smallest quantity of complement which produces complete hemolysis. This smallest quantity is then taken as its test value. For instance, if smallest quantity is 0.03, then to 97 c. c. NaCl solution 3 c. c. complement is added; if it is 0.02, only 2 c. c. is added to 98 c. c. NaCl solution.
3	2.6	.4	1	1	
4	2.7	.3	1	1	
5	2.8	.2	1	1	
6	2.9	.1	1	1	
7	3.5	.5	-----	1	Complement control (no hemolysis should occur).
8	3.0	-----	1	1	Amboceptor control (no hemolysis should occur).
9	4.0	-----	-----	1	Salt-solution control (no hemolysis should occur).

¹ 0.85 per cent NaCl solution.² Guinea pig serum in diminishing quantities.³ Of previously titrated hemolytic serum, double dissolving quantity.⁴ 5 per cent suspension of sheep blood corpuscles washed in salt solution.⁵ This amount is taken from the basic dilution in tube 1.

The amboceptor should be inactivated when used fresh; that is, before it is three days old. Otherwise it is carbolized with 10 per cent of 5 per cent carbolic-acid solution, and kept in ice box—in this case it is used without inactivation.

Place tube rack in incubator for 2 hours and read results.

This test should be made as a preliminary to every suspected glanders serum test, as it is always necessary to determine the smallest quantity of complement to be used for the final test. Of course, within 24 hours any number of tests can be made with the same complement dilutions.

SPECIFIC COMPLEMENT FIXATION (DEVIATION).

Complement fixation is a biological reaction in which the phenomenon of hemolysis is employed as the fundamental principle. It is so called on account of the fact that the complement has been fixed by the combination of antigen with antibody and thus prevented from participating in the hemolytic process in which it is essential in order to have hemolysis. By this method even small quantities of amboceptors (antibodies) can be demonstrated in a serum.

The presence of an infectious principle in the organism of an animal or a man has a stimulating effect on the production of antibodies (immune bodies). If a serum containing such immune bodies is inactivated and brought into contact with the antigen in the presence of complement, the complement will become firmly fixed by the combined immune body and antigen. (See Pl. I, B.) Thus, anchoring takes place between the antigen and the antibody in which the complement becomes fixed. This anchoring is thoroughly established when the mixture is placed in an incubator for one hour. The addition of the hemolytic amboceptor and blood corpuscles to

TITRATION OF COMPLEMENT (GUINEA-PIG SERUM).

(Tube 3 represents the smallest quantity of complement producing complete hemolysis.)

such an anchored antigen and immune body will have no effect. (See Pl I, D.) Thus, no hemolysis will take place, inasmuch as the complement has been fixed by the immune body and the antigen, thereby leaving the hemolytic system incomplete. On the other hand, if the inactivated serum contains no immune bodies, there would be no substance in the serum to anchor the antigen. As a result, therefore, no fixation of complement will occur, this being left free, and on addition of hemolytic amboceptor and blood corpuscles, hemolysis will now take place. (See Pl. I, C.) Neither the antigen nor the antibody alone can fix the complement and thereby influence hemolysis when the hemolytic amboceptor and blood corpuscles are added. However, in combination the fixation will invariably take place, and on the addition of the hemolytic amboceptor and blood corpuscles hemolysis will not be produced.

Since the discovery of this phenomenon it has been utilized extensively in serum diagnosis, but probably its greatest value has been obtained from the Wassermann reaction for the diagnosis of syphilis. It has also been employed in other diseases with more or less satisfaction, and its great field in bacteriological investigations has not yet been exhausted for the practical diagnosis and determination of immune bodies in serum. In veterinary practice complement fixation is now gradually becoming used for the diagnosis of glanders. This method of diagnosing glanders has given the most favorable results in Germany, and constitutes at the present time the official test for Prussia and other parts of Germany. It has also been used in the diagnosis of other diseases of animals, but not with such success as in glanders. Particularly in tuberculosis the results were not uniform and otherwise not very promising.

The presence of the specific immune bodies (bacteriolytic amboceptors) in the serum of glandered horses brings about the fixation of the complement when the antigen in the form of glanders bacilli extract is added to the hemolytic system. The serum of glandered horses, therefore, contains antibodies (immune bodies) against glanders bacilli, which are specific only for the glanders bacilli and for no other infection. The complement fixation accordingly represents a specific test, as only in the presence of the glanderous immune bodies and glanderous antigen will the reaction take place. If, instead of the glanderous immune bodies, other antibodies of another infectious disease be present in the blood serum, they will exert no effect whatsoever on the glanderous antigen; and, on the other hand, if serum containing glanderous immune bodies is brought in contact with an antigen of another infectious disease, it will also have no effect on the reaction. By this fixation of the complement the hemolytic system is left incomplete, and as a result no hemolysis will take place. This fixation of the complement by the antigen and immune

bodies of glanders in the horse serum constitutes the diagnostic test for this disease.

In the application of the test it is necessary to have substances constituting the hemolytic system, which are the washed blood corpuscles of a sheep, the hemolytic amboceptor (rabbit serum), and complement (normal guinea pig serum). The quantity of the hemolytic amboceptor to be used has been established by titration and described in a preceding part of this publication. In the test for glanders the double strength of the determined titre of the hemolytic amboceptor is used. A slight excess in the quantity of the amboceptor does not alter the outcome of the tests.

On the other hand, the establishment of the smallest amount of complement which will produce hemolysis in the presence of the hemolytic amboceptor and blood corpuscles is very essential, and accordingly this should be established by the previously described preliminary test, before tests for glanders are undertaken. By omitting this it is possible that an excessive amount of complement would be used which would in some cases affect the final results of the test. An oversupply of complement in the test may not only prove sufficient to be fixed by the immune bodies (antibodies) and antigen, but there might be also enough complement left to produce an incomplete or an almost complete hemolysis. Thus it is evident that it is of great importance to establish by such a preliminary test (see Table 2) the exact quantity of complement to be used. Schütz and Schubert found that the success of this method of diagnosing glanders depended greatly upon the proper quantity of complement used, and therefore the establishment of this quantity should not be omitted.

The red blood corpuscles of the hemolytic system always constitute a uniform quantity—that is, a 5 per cent suspension of the washed corpuscles in salt solution. As has been stated, this may be kept for testing purposes for two or even three days in the ice chest. The method of obtaining the corpuscles has been described in the early part of this work.

In the complement-fixation test, there are also used, besides the hemolytic system, the serum of the horse to be examined and antigen.

METHOD OF OBTAINING SERUM OF ANIMALS TO BE TESTED.

The blood is drawn from the jugular vein of the suspected horse after a small area over the jugular has been clipped and disinfected with alcohol. The vein is dilated by pressure on the lower part of the neck, and the blood is drawn from the animal by the insertion of a trocar and cannula. It is recommended that the blood should be collected in uniform-sized tubes or bottles, which should be sterilized before using. A sufficient quantity of blood for testing purposes

would be 50 to 100 c. c., and after allowing the blood serum in the tube to separate from the clot in a cool, dark place it is ready to be used for the test. If it is desired to forward the blood to a laboratory the tubes may be packed into separate containers or collectively in a box. Every tube should be labeled, and the number of the horse corresponding with the record number should be designated on the label. It is not absolutely essential to have clear serum, as in repeated tests carried out in this laboratory it was found that blood forwarded from Michigan to Washington gave satisfactory reactions, although the serum was badly discolored as a result of disintegration of the blood corpuscles. If it is desired to preserve the serum, or if from some cause a test can not be applied during the first few days after the blood has been drawn, it should be preserved with a 0.5 per cent solution of carbolic acid. This percentage is best obtained by adding 1 part of a 5 per cent carbolic-acid solution to 9 parts of the serum to be preserved. Such carbolized serum will respond to this test after several months.

In cases where the mallein test has been used the blood of suspected horses to be examined for glanders by complement fixation should not be taken until from 7 to 10 days have elapsed after the last mallein test. This is necessary because of the possibility that the injected mallein may have exerted a stimulating effect on the cells with the production of immune bodies, and if serum is then taken for the test the results may lead to a faulty diagnosis. However, in suspected cases of glanders the blood of the animals may be drawn and forwarded for examination to a laboratory where the serum diagnosis of glanders is practiced, without the necessity of the application of the mallein test.

INACTIVATING THE SERUM.

Of the horse serum to be examined about 2 c. c. is drawn off and placed in a suitable tube or bottle in order to subject it to a temperature of 58° C. in a water bath for one-half hour. This constitutes the inactivation of the serum: that is, the complement which is present in the serum is destroyed by this heating. Such inactivated serum is ready for use in the testing, but should be used only on the day of its inactivation. In case it becomes necessary to repeat the test, another 2 c. c. of the sample should be inactivated.

The method of inactivation referred to above applies only to horses. Miessner and Trapp found that serum of mules inactivated at 58° C. and 59° C. does not in all cases give satisfactory results, as in many instances even the normal serum of these animals checks hemolysis. The numerous tests which were carried out in this laboratory with serum of horses and mules proved that while the inactivation of the horse serum at 58° C. and 59° C. always gave satisfactory results, the tests made with mule serum under the same conditions were far from uniform.

Accordingly, it was deemed advisable to inactivate sera from mules at a higher temperature as well as by carbolization. Normal mule serum was subjected to various degrees of temperature ranging from 58° C. to 62° C. for three-fourths to one hour, and similar samples of serum were carbolized and inactivated at the same temperature as the noncarbolized serum. The results showed that carbolized mule serum inactivated at 60° C. gives the most uniform results in the complement-fixation test. The same serum if not carbolized showed checking of hemolysis at an inactivation recommended for horse serum. Inactivations at a higher degree of temperature are not advisable, inasmuch as at 62° C. a considerable percentage of sera will coagulate. Therefore, in testing mule sera a carbolization with 0.5 per cent carbolic acid followed by an inactivation at 60° C. for one hour is advisable.

The antihemolytic action of the normal sera of mules is probably due to the presence of substances in the blood which exert a checking action on hemolysis, as glycogen, pepton, albumose. These undetermined substances act by using up the complement, having a greater affinity toward it than the hemolytic amboceptor has.

PREPARATION OF THE ANTIGEN (GLANDERS BACILLI EXTRACT).

The antigen represents a shake extract of glanders bacilli in salt solution. It is prepared as follows: From a stock culture of glanders bacilli subcultures are made on 2 per cent acid glycerin agar media. It is preferable to use Kolle flasks instead of tubes for the cultures, inasmuch as in such flasks the surface of the media is much larger and a greater quantity of bacilli can be obtained from them. After inoculating the media with glanders bacilli the flasks are placed in the incubator, and after 24 hours the condensation water in the cultures is allowed to run over the surface of the media. After another 24 or 48 hours in the incubator the surface of the media contains usually a luxuriant growth of glanders bacilli, and it is then ready for washing. To each flask 20 to 40 c. c. of salt solution is added. If the cultures have been grown in ordinary test tubes they are washed off with 5 to 15 c. c. of salt solution.

After the entire growth is washed off the surface of the medium the fluid is poured into sterile flasks and then heated to 60° C. for four hours in order to kill the glanders bacilli. After the heating of the bacilli the flasks are placed in a shaking apparatus and shaken for four days.

After removal of the flasks the extract is placed in centrifuge tubes and centrifugalized for two hours in a centrifuge at a speed of 2,500 to 3,000 revolutions per minute. Then to the clear liquid, which has been drawn off into suitable bottles, 10 per cent of a 5 per cent carbolic-acid solution is added, and the bottles are corked. This rep-

resents the extract or antigen to be used for the tests in a dilution which is established by titration. The extract usually keeps for two or three months, or sometimes even longer if kept in a dark, cool place.

TITRATION OF THE EXTRACT.

The titration of the extract is carried out in order to establish the quantity of the extract which no longer prevents hemolysis. First, dilutions with salt solution are made from the extract in proportions of 1 to 20, 30, 40, 50, and so on up to 200. These dilutions are made from a basic dilution of 1 to 10, as can be seen from Table 3. The titration proper is carried out as follows: Nine test tubes are used for the titration and three for controls. To each tube 1 c. c. of salt solution is added, then 1 c. c. of complement of the determined smallest quantity established according to the preliminary test (see Table 2). This is followed by the extract, each tube receiving 1 c. c. of the different dilutions prepared. Thus, in the first tube 1 c. c. of the dilution of 1 to 10 is added; in the second, 1 c. c. of the dilution of 1 to 20; in the third, 1 c. c. of the dilution of 1 to 30, and so on. Then the rack is placed in the incubator for one hour. After it is taken out, 1 c. c. of a double quantity of the previously titrated amboceptor is added to each tube, and finally 1 c. c. of a 5 per cent suspension of washed sheep blood corpuscles. Of the three control tubes, the first serves for a control to show that the complement alone (without the amboceptor) does not produce hemolysis, the second to show that the amboceptor alone does not produce hemolysis, and the third that the salt solution alone does not produce hemolysis. Thus, in the first control tube 3 c. c. of salt solution, 1 c. c. of complement, and 1 c. c. of blood corpuscles are added. The second tube contains 3 c. c. of salt solution, 1 c. c. of amboceptor, and 1 c. c. of blood corpuscles, while the third contains 4 c. c. of salt solution and 1 c. c. of blood corpuscles. This titration is similar to the titration of the hemolytic amboceptor.

After shaking the test tubes, which should invariably follow all these additions, the rack is placed in the incubator for two hours. Then the results are read. The tube in which hemolysis is no longer prevented represents the titre of the extract. (See Pl. IV.) In the glanders test, however, one-half of that quantity is used. Thus, if the titration should prove that the first tube which does not prevent hemolysis contains a dilution of 1 to 50, then a dilution of 1 to 100 of the extract is used for the glanders tests.

In Table 3 the titration of the glanders bacilli extract is given in consecutive order, and this may be followed in a similar manner as with the other tables. In this table the substances to be used are given consecutively, and likewise the exact quantities are indicated.

TABLE 3.—*Titration of glanders bacilli extract.*

Tube.	NaCl solution. ¹	Comple- ment. ²	Extract. ³		Ambo- ceptor. ⁴	Blood cor- puscles. ⁵	Remarks.
	c. c.	c. c.	1 c. c. of—		c. c.	c. c.	
1	1	1	1:10...	Place for 1 hour in incubator.	1	1	One-half of the quantity of the extract which no longer prevents hemolysis is the proper value to be used in future final testing. Thus, if it should show by this test to be 1 to 50, then a dilution of 1 to 100 of the extract is used.
2	1	1	1:20...		1	1	
3	1	1	1:30...		1	1	
4	1	1	1:40...		1	1	
5	1	1	1:50...		1	1	
6	1	1	1:60...		1	1	
7	1	1	1:80...		1	1	
8	1	1	1:100...		1	1	
9	1	1	1:200...		1	1	
10	3	1	-----		-----	1	Complement control (no hemolysis should occur).
11	3	-----	-----		1	1	Amboceptor control (no hemolysis should occur).
12	4	-----	-----		-----	1	Salt solution control (no hemolysis should occur).

¹ 0.85 per cent NaCl solution.² The determined smallest quantity established according to the preliminary test.³ A basic dilution is made from the extract in proportions of 1 to 10, 20, 30, etc., up to 200. From these basic dilutions the third column is made as indicated below.⁴ Double the quantity previously determined by titration.⁵ 5 per cent suspension of sheep-blood corpuscles washed in salt solution.

The basic dilutions of the glanders bacilli extract are made as follows:

A dilution of—

- 1 to 20 is made by adding 1 c. c. of 1 to 10 dilution to 1 c. c. NaCl solution.
- 1 to 30 is made by adding 1 c. c. of 1 to 10 dilution to 2 c. c. NaCl solution.
- 1 to 40 is made by adding 1 c. c. of 1 to 10 dilution to 3 c. c. NaCl solution.
- 1 to 50 is made by adding 1 c. c. of 1 to 10 dilution to 4 c. c. NaCl solution.
- 1 to 60 is made by adding 1 c. c. of 1 to 10 dilution to 5 c. c. NaCl solution.
- 1 to 80 is made by adding 1 c. c. of 1 to 10 dilution to 7 c. c. NaCl solution.
- 1 to 100 is made by adding 1 c. c. of 1 to 10 dilution to 9 c. c. NaCl solution.
- 1 to 200 is made by adding 1 c. c. of 1 to 100 dilution to 1 c. c. NaCl solution.

THE COMPLEMENT-FIXATION TEST.

As previously stated, the serum of glandered horses contains immune bodies which develop during the course of the disease. The presence of these immune bodies in the serum is utilized in the development of the test. Naturally, the amount of these immune bodies in the affected horses is not uniform and undoubtedly depends on the extent of the infection present; therefore it is advisable to use in the test such quantities of the serum as will prove sufficient for the reaction to take place.

The necessary quantity for the test has accordingly been established, through the painstaking experiments of Shütz and Schubert, as 0.2 and 0.1 c. c., placed in two different tubes. In some instances the tube containing 0.2 c. c. of the serum may show a fixation of the complement, while the tube containing 0.1 c. c. of the same serum may show only a partial fixation or hemolysis. In the great majority of cases, however, the fixation is usually manifested in both tubes.

The substances which are used in the fixation test have already been described in detail, their strength has been established by the titration of these different substances, and it need only be mentioned in passing that the best results will be obtained when the smallest quantities of substances permitted by the titration are used for the test; that is, the substances should have a high titre. If, for instance, the glanders extract should prove of a low titre, the albumins which are contained therein may have an influencing effect upon the outcome of the test, inasmuch as it is known that albumins or cell extracts, if present in large quantities, will fix complements. Therefore it is always advisable to use glanders extract of a high titre; the quantity to be used should not be much above 0.01 c. c.

Besides the centrifugal machine and an ordinary shaking apparatus, only glassware is needed for the test. This consists principally of pipettes of various sizes, and there should always be a sufficient number on hand during the performance of the test. Those best adapted for the work are 1 c. c. pipettes, graduated into hundredths, and 10 c. c. pipettes, graduated into tenths. In addition, several pipettes of various sizes will be found useful for measuring the various substances to be diluted. Uniform-sized test tubes are advised and they should fit into test-tube racks. All the glassware should be cleaned and dry-sterilized before using. Only such a number of tests as can be conveniently handled should be undertaken on a single day. Finally, the incubator should contain sufficient space to hold the necessary number of racks during the test. With a good-sized incubator and the above-mentioned apparatus any State bacteriological laboratory could meet the demand for diagnoses from all

the veterinarians within its borders, as from 80 to 100 tests may readily be made by this method in one day.

APPLICATION OF THE TEST.

Before undertaking the tests it is advisable to prepare the dilutions which will be used for the testing. The quantities of dilutions can be determined quite accurately, in accordance with the number of tests to be made. Thus the necessary quantity of a suspension of blood corpuscles is prepared, as well as the dilution of the necessary quantity of the amboceptor, complement, and glanders extract. The preparation of these dilutions is best accomplished in Erlenmeyer flasks, and the dilutions prepared should not be kept over one day.

Four test tubes are used in testing the serum of each horse. For convenience in reading the results they are placed in a test-tube rack. The first test tube is marked with the number of the animal corresponding with that on the record for identification. Thus, for instance, should it be necessary to examine 20 horses, 80 tubes would be required for the tests. The first and third tubes are intended for the test proper, while the second and fourth tubes serve as controls for the horse serum to be examined. All the test tubes are first filled with the necessary quantity of salt solution, which will make up with the other substances used the required 5 c. c. Thus the first and third tubes each contain 1 c. c. of salt solution, while the second and fourth tubes contain 2 c. c. each. Then the suspected horse serum to be examined, which has been previously inactivated by heating for one-half hour at 58° C., is added. In the first and second tubes 0.1 c. c. and in the third and fourth tubes 0.2 c. c. of this inactivated serum is added. This is followed by the addition of the glanders bacilli extract, which, however, is only introduced into the first and third tubes, 1 c. c. of the established dilution being used. The second and fourth tubes, therefore, do not contain glanders bacilli extract, as these tubes serve only as controls to show that the horse serum alone will not influence hemolysis. Thus in every test the amount of serum used in the tests proper (first and third tubes) is controlled by an equal amount of serum (second and fourth tubes).

After the addition of the antigen (glanders extract), 1 c. c. of the complement is added to every tube in a dilution which has been established by the preliminary tests. (See Table 2.)

At this stage of the testing each tube contains 3 c. c. of fluid. The rack containing the tubes is now shaken and placed in the incubator for one hour. This incubation is carried out in order to allow the anchoring of the antigen and the glanders immune body, in which the complement will become firmly fixed. Naturally, the anchoring

would only take place provided the serum contains the glanders immune bodies; that is, when the horse is infected with glanders. (See Pl. I, D.) On the other hand, in the absence of a glanders infection the serum does not contain immune bodies, and therefore the antigen as well as the complement will remain free in the test tubes. (See Pl. I, C.)

After the time required for the incubation has elapsed the rack is removed and the other substances of the hemolytic system are added, namely, the hemolytic amboceptor and the blood-corpuscles suspension.

To each test tube is added 1 c. c. of the previously titrated rabbit serum (hemolytic amboceptor), and finally 1 c. c. of a 5 per cent suspension of sheep blood corpuscles which have been previously washed with salt solution. The tubes, either individually, or collectively in the rack, should then be well shaken by hand, in order to mix the fluids thoroughly and to wash down any flecks of the solutions that might have adhered to the sides of the tubes.

This practically concludes the test, and it is now only necessary to incubate the tubes for 10 hours, when the results may be read.

CONTROLS.

During the application of all tests control tubes are also used at the same time for comparative purposes. Four tubes are required for the control of the hemolytic system and two for the control of the glanders extract. The control of the hemolytic system is established by using in the first tube all substances constituting the hemolytic system, namely, the hemolytic amboceptor, the complement, and the suspension of blood corpuscles. This tube after the conclusion of the test should show a complete hemolysis. In the second tube the hemolytic amboceptor is controlled without the complement, to prove that it does not produce hemolysis. The third tube serves as a control of the complement without the hemolytic system, also to prove that it does not produce hemolysis. The fourth tube is to show that the salt solution has no hemolytic action on the blood corpuscles. The quantities of the different substances used in the controls are the same as used in the regular tests for glanders. It is necessary in the control tubes, as well as in all other tubes, to make up the quantity to 5 c. c. with salt solution. Thus, in the tube which serves for the control of the hemolytic system, 2 c. c. of salt solution is added, while in the control of the hemolytic amboceptor as well as in the control of the complement 3 c. c., and in the salt-solution control 4 c. c., are added. The substances added to the control tubes are

used in the same order as given in the test, and the incubation is also followed at the same time.

As above stated, two test tubes are used for the control of the antigen (glanders bacilli extract), in addition to the four used for the control of the hemolytic system. These two tubes contain the hemolytic system, and to the first tube 1 c. c. of the extract used in the glanders test is added, while to the second tube is added 2 c. c. of the extract. The controls of the extract serve to prove that the extract used in the tests does not cause a fixation of the complement, and that even double the quantity of the extract which has been used in the glanders test does not fix the complement. In the extract controls we have in the first 1 c. c. of salt solution, while to the second tube no salt solution is added, inasmuch as the substances in this control make up the necessary 5 c. c. In the first tube, besides the 1 c. c. of salt solution, 1 c. c. of the extract is added, which is followed in consecutive order with the substances of the hemolytic system, in quantities and dilutions used during the test proper, which have been previously indicated.

The results in the control tubes after the conclusion of the tests should show the following:

All the control tubes which contain, besides the hemolytic system, only serum of the suspected horse—that is, the second and fourth tubes—should show a complete or almost complete hemolysis. There should be no deposit of blood corpuscles on the bottom of these tubes, or only a very small quantity. The fluid should have the characteristic (laked) wine-red color.

In the control tubes of the extract there should also be a complete or almost complete hemolysis. The same should be present in the control of the hemolytic system; it should show a complete hemolysis. In the control tubes of the complement the hemolytic amboceptor, and the salt solution, there should be no hemolysis whatever; that is, the blood corpuscles should be settled in the bottom of the tube, and the liquid above them should be water-clear.

In Table 4 the final test for glanders is given in the order in which it should be undertaken. The number of test tubes to be used is indicated, and also the number of controls to be made. The substances and quantities, as well as the dilutions, are also indicated in the explanatory remarks of the table. As already stated, six controls are sufficient for any number of tests undertaken on one day, if the same substances are used for all the tests. In making the complement-fixation test it is always advisable to control the results further by using serum from an established case of glanders in one series of four tubes, and from a healthy horse in another similar series, for comparative purposes.

FINAL TEST SHOWING POSITIVE REACTION TO GLANDERS.

Tubes 1 and 3 show fixation of complement indicative of glanders.
Tubes 2 and 4, controls for the suspected horse serum.
Tubes 5 and 6, controls for the antiserum.

Tube 7, control for hemolytic system.
Tube 8, control for complement.
Tube 9, control for hemolytic amboceptor.
Tube 10, control for salt solution.

TABLE 4.—*The final test for glanders.*

Tube.	NaCl solution. ¹	Suspected horse serum. ²	Glanders bacilli extract. ³	Complement. ⁴		Amboceptor. ⁵	Blood corpuscles. ⁶	Remarks.
1	c. c. 1	c. c. 0.1	c. c. 1	c. c. 1	Place for 1 hour in incubator.	c. c. 1	c. c. 1	Test tube for the dose 0.1 c. c. of suspected serum.
2	2	.1		1		1	1	Serum control for the dose 0.1 c. c. of suspected serum.
3	1	.2	1	1		1	1	Test tube for the dose 0.2 c. c. of suspected serum.
4	2	.2		1		1	1	Serum control for the dose 0.2 c. c. of suspected serum.
5	1		1	1		1	1	Control for the quantity of extract used (hemolysis).
6			2	1		1	1	Control for the double quantity of extract, for greater accuracy (hemolysis).
7	2			1		1	1	Control of the hemolytic system (hemolysis).
8	3			1			1	Control of the complement (no hemolysis).
9	3					1	1	Control of amboceptor (no hemolysis).
10	4						1	Control of salt solution (no hemolysis).

¹ 0.85 per cent NaCl solution.² Suspected horse serum to be inactivated for 30 minutes at 58° C. in water bath, in order to destroy the complement which is present in the serum of every horse.³ One-half of the quantity which does not prevent hemolysis and established by titration. (See Table 3.)⁴ The determined smallest quantity established according to the preliminary test. (See Table 2.)⁵ Double the quantity previously determined by titration. (See Table 1.)⁶ 5 per cent suspension of washed sheep-blood corpuscles.

INTERPRETING RESULTS OF TESTS.

The results of the tests are manifested in most instances by a distinct reaction which takes place in the test tubes. The indication of the results is only to be sought in the tubes numbered 1 and 3, which represent the actual tests for the presence or absence of glanders, inasmuch as these tubes contain all substances required in the tests.

We may thus obtain in these tubes either complete hemolysis, incomplete hemolysis, or no hemolysis whatsoever. The fixation of the complement is manifested by the absence of hemolysis, and therefore we have in the first and third tubes a settling of the blood corpuscles with the watery clear fluid above. Such a result indicates without doubt the presence of glanders. On the other hand, if the first and third tubes show complete hemolysis the absence of glanders is thereby indicated. In the presence of glanders a fixation of the complement takes place, as a result of anchoring to the immune bodies and antigen (see Pl. I, D), while in the absence of glanders, there being no immune bodies present, the complement is used up in the phenomenon of hemolysis (see Pl. I, C).

Then, again, we may have cases in which the fixation of the complement is incomplete; that is, there is a settling of corpuscles in the bottom of the test tube, but the fluid shows traces of hemolysis. It

does not show the characteristic saturated color of hemolysis, but only a tingeing with the hemoglobin. This is termed an almost complete fixation, and also indicates the presence of glanders. The presence of the characteristic color in the fluid and a very slight deposit of corpuscles on the bottom should not be taken as an indication of an infection, as such a condition may be brought on by various causes, and particularly so by the presence of nonspecific substances in the serum of the horse, which may cause a very slight checking of the hemolysis. But all cases where the results show a fixation of the complement (no hemolysis) or almost complete fixation (slight tingeing of the fluid above the settled blood corpuscles) indicate the presence of glanders.

As a result of the different quantities of horse serum used in the two pairs of test tubes, it will be found occasionally that the test tube containing the larger quantity of serum (0.2 c. c.—third tube) shows a fixation of the complement, while the tube containing the smaller quantity of serum (0.1 c. c.—first tube) shows a partial fixation of the complement. This also can be considered as a glanderous infection, as it shows that there are present immune bodies, but not to such an extent as to produce the fixation of the complement in the tube where the smaller quantity of the suspected horse serum has been used. In most instances of glanderous infections, however, it will be observed that the fixation of the complement is uniform and almost complete in both tubes.

The reading should be made after the results in the test tube have been clearly established, and one should not be too hasty in drawing conclusions before the reaction in the test tubes is clear enough to establish the results of the test.

In the illustrations in Plate V the results of the glanders tests are indicated by the presence of a positive reaction. In tubes 1 and 3 a complete fixation of the complement has taken place, there being no hemolysis, while tubes 2 and 4 represent the controls for the horse serum (no glanders extract being added), these tubes showing hemolysis. In the upper row of the rack the controls are indicated, and they show the results as they should be obtained in the course of testing.

Should the horse prove glandered on the above test, the test may be repeated, and then serum quantities are used as follows: 0.1, 0.05, 0.03, 0.02, and 0.01 c. c. One control is made of 0.1 c. c. as in tube 2 of the test. By this method the smallest quantity of serum is established which will divert the complement, but it is not necessary as routine practice.

Our own experience, as well as the work of Schütz and Schubert, to whom we wish to extend our acknowledgments, shows that the results of the complement-fixation test should be interpreted as follows:

1. Horses in which the serum produces a complete fixation of the complement in the quantities of 0.1 c. c. and 0.2 c. c. should be considered as glandered.

2. Horses in which the serum gives a complete fixation in the quantity of 0.2 c. c. and an incomplete fixation in the quantity of 0.1 c. c. should likewise be considered as glandered.

3. Horses in which the serum produces an incomplete fixation of the complement in the quantities of 0.1 c. c. and 0.2 c. c. should also be considered as glandered.

4. Horses in which the serum shows no fixation of the complement in either tube should be considered free of glanders.

In order to reduce the possibility of error to a minimum the agglutination test may be applied to the latter cases, and if this shows a value of 1 to 1,000 or over, the animal should be considered as glandered. However, such cases are extremely rare.

The agglutination test may be undertaken with the complement-fixation test without a great deal of difficulty, especially if the agglutination test is carried out in accordance with the method used at the present time in Germany, by which the results of the test can be obtained in about two hours. This consists of a modification of the agglutination test, in which by centrifugalization the agglutination is hastened in the test tubes and the results can be read after the tubes have been placed in the incubator for two hours.

The technique of this method of agglutination testing will not be explained here, but it is planned to describe it in a later publication on this subject.

CONTROLLING GLANDERS IN AN INFECTED STABLE.

When glanders is discovered or suspected among the horses in a stable, the blood of all the horses in the infected stable should be drawn and tested in the manner previously described. All animals whose serum shows a complement fixation should be destroyed without further consideration. After the animals have been killed the stable should be thoroughly cleaned and disinfected. The animals which gave no reaction on the first test should be retested after three weeks, and should there be no indication of the disease in the second test the stable may be considered free from the infection. On the other hand, if on the second test one or more animals should give a reaction, the infected animals should be destroyed and all the remaining horses should again be subjected to another retest after three weeks. Not until the last test proves that no animal is affected can the stable be considered as free from the infection.

RESULTS OF PRACTICAL TESTS WITH COMPLEMENT FIXATION.

In order to ascertain how long a time after infection with glanders elapses before the presence of the disease could be detected by the complement-fixation test, a horse was infected with glanders at the Bureau of Animal Industry Experiment Station by taking a

loopful of glanders culture and rubbing it up and down the Schneiderian membrane. Twenty-four hours after the infection the first sample of blood was taken from the jugular, and this was followed by taking samples daily for about a month. The horse was inoculated on January 17, and the blood which was taken on January 22 gave a positive reaction to the complement-fixation test, thereby proving that five days after infection the presence of glanders could be ascertained by complement fixation. The animal at that time showed no clinical indication of the disease, but three days later (on the eighth day) the discharge from the nose appeared, and at the same time the characteristic swelling of the submaxillary lymph glands was noted. Repeated tests made with blood taken daily from the horse gave always the characteristic reaction. After four weeks from the time of the infection the reaction appeared to be less pronounced, this probably being due to the acuteness of the disease and the great amount of toxin which had been produced continuously in the animal. The testing of this horse was not continued after the disease had affected the animal to such an extent that it appeared dangerous to continue the work, owing to the extensive infection accompanied by a very profuse discharge.

Since this method of diagnosis for glanders was inaugurated in this laboratory, large numbers of horses and mules have been examined in the District of Columbia, as well as animals from other parts of the United States. Many of the horses examined had clinical cases of glanders, while others were selected because they were reactors to the mallein test, some typically, and others atypically. A large proportion of the cases, however, were exposed or "contact" horses. From the number of tests already made—about 1,540—the results indicate that in the complement fixation we have a method which in accuracy is equal to the tuberculin test for the diagnosis of tuberculosis in cattle. The results of the tests thus far conducted show that at least 97 per cent of the cases of glanderosus affections can be determined by the complement-fixation method. Furthermore, the affected horses in which a negative or atypical reaction occurs are as a rule either very old chronic cases of glanders, or those fresh cases of infection tested during the period of incubation. According to Hutyra and Marek,¹ the diagnosis of glanders by the complement-fixation test has already given such accurate results that it may be considered as the best method for the determination of this disease at the present time.

A good opportunity was recently afforded to apply the test extensively in an outbreak of glanders which occurred during the month of January, 1911, in a fashionable boarding stable in the District of

¹ *Spezielle Pathologie und Therapie der Haustiere.* Third edition, Band 1, 1910, p. 717.

Columbia. At this stable two clinical cases of glanders were found, and the physical examination of 60 horses in the stable showed three additional cases with not typical but somewhat suspicious symptoms. The testing of all the horses in the stable was immediately undertaken, and as a result of the complement-fixation tests 14 horses were found to be affected with glanders. Unfortunately it was impossible to obtain the consent of the proprietor to test the horses with mallein for a comparative study of these two methods of diagnosis.

Careful post-mortem examinations were made on all the reacting animals, and in every instance lesions of glanders were found in one or more organs or tissues. In some of the cases only a few characteristic pulmonary nodules were present, while in others the characteristic lesions of acute glanders of the lungs and liver were present. There was not a single case among these horses in which the diagnosis on post-mortem examination could have been doubted.

After removing and killing the affected animals the stable was thoroughly cleaned and disinfected, and a second test was made with the blood of the remaining horses three weeks from the time the first test had been conducted. On the second test all the horses except one gave an absolutely negative reaction. This horse was destroyed at once, and the post-mortem examination revealed a caseated focus in the left bronchial lymph gland and about twenty small nodules of glanders in the lungs, all of which were evidently recent, having developed without doubt during the interval between the tests, as a result of the previous heavy exposure.

In all other cases where the test has been applied and in which careful post-mortem examinations were held after the destruction of the positive cases the presence of glanders has been demonstrated. Of course not all the animals which gave a reaction were autopsied, inasmuch as in some instances the tests were made for State officials, the animals not being under the jurisdiction of the Department of Agriculture. Again, in other cases where the animals were destroyed, no data of the post-mortem examinations were furnished.

Among the horses tested by the complement fixation there were a number of animals which gave an atypical reaction to the mallein test, but on the complement-fixation test they proved either absolutely positive or negative. Of these horses those which gave a positive reaction and were killed proved to be glandered.

Table 5 shows the comparative results obtained with the mallein and complement-fixation tests:

TABLE 5.—*Comparative results with mallein and complement-fixation tests.*

Locality.	Positive to mallein test.				Negative to mallein test.			Atypical reaction to mallein test.				Total animals.		
	Total.	Response to complement-fixation test.		Post-mortem.	Total.	Response to complement-fixation test.		Total.	Response to complement-fixation test.		Post-mortem.			
		Positive.	Negative.			Positive.	Negative.		Positive.	Negative.			Positive.	Negative.
Connecticut.....	9	8	1	3					1		1		10	
Florida.....	9	5	4		9	8	1	6		6			24	
Illinois.....	6	4	2					4	4				10	
Indiana.....	5	3	2		11	2	9	3	1	2	1		19	
Maine.....	7		7	7	32		32	12		12		3	51	
Michigan.....	18	17	1		3		3	6		6			27	
Missouri.....	1	1						9	3	6			10	
Montana.....	35	26	9		17	4	13	8	4	4			60	
North Dakota.....	19	19			11	11		12	6	6			42	
Pennsylvania.....	1		1					1		1			2	
Nebraska.....	6	5	1					6	3	3			12	
Wyoming.....	5	5						2		2			7	
Washington.....	2	2						1	1				3	
California.....	6	4	2										6	
Texas.....	5	2	3					4		4			9	
Oregon.....	2	2						1	1				3	
Canada.....	1		1										1	
Miscellaneous.....								29	20	9	20		29	
Total.....	137	103	34	3	7	83	25	58	104	44	61	22	3	325

In addition to the above there have been 1,217 tests made upon horses with the complement-fixation method alone, and we have also tested by this method the blood of one lion, which gave a positive reaction, and the blood of one human suspected of having glanders, which proved negative. These two results were later substantiated.

Of the above-mentioned 1,217 tests, 643 were conducted on horses at Washington, D. C., and of these 21 gave a positive reaction, all of which were subsequently confirmed on post-mortem. The remaining 575 were from miscellaneous sources, 78 of which were positive, while 497 were negative.

In the 323 cases in Table 5 wherein the two tests are compared, the mallein test was confirmed by the complement-fixation test in 161 cases and was not confirmed in 59 cases. There were 104 atypical reactions to mallein which were definitely diagnosed by complement fixation—44 positive and 60 negative. Seven of the Maine reactors to mallein and three atypical reactors have been examined post-mortem without showing any evidence of glanders.

In order to determine whether the fixation of the complement may be obtained occasionally in normal horses or in horses affected with various diseases other than glanders, a number of tests were made with the blood of apparently normal horses and, also, with horses suffering with various infectious and noninfectious diseases. One of these tests was made with the blood of a horse affected with swamp fever, in which the temperature registered 106.2° F.; other tests were made with blood from horses affected with distemper, dourine, influenza, pneumonia, heaves, lameness, fistulous withers, forage poisoning, etc.; but in all these cases negative reactions were obtained.

From these results the specificity of the test can be readily appreciated. Animals affected with glanders will give a positive reaction. Normal animals or those affected with diseases other than glanders will give no reaction.

LIBRARY OF CONGRESS

0 002 824 810 6