

THE ANTIGENIC VALUE OF SPIROCHÆTA HYOS IN COMPLEMENT-FIXATION TESTS ON HOG- CHOLERA SERA *

STUDIES ON HOG CHOLERA

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No references to laboratory methods for the diagnosis of hog cholera occur in the earlier literature with the exception of that to the use of *Bacillus cholera-suis* in experimental agglutination tests.¹ Within the last few months reports have been made of the results of some experimental complement-fixation reactions.

Connaway² and his associates found that antigen prepared from the blood of pigs suffering from hog cholera was unsatisfactory. Negative results also followed the use of antigens prepared from the spleens and kidneys of virus pigs.

Healy and Smith³ have published results obtained with an antigen prepared from the mesenteric glands of cholera hogs. This was made by grinding 18 gm. of selected mesenteric-gland tissue with sterile sand. To this, 180 gm. of neutral 1% glucose broth were added, and the mixture allowed to stand for 8 days at 4 C. The results of tests with this material led the authors to conclude that they had obtained "an antigen which shows striking differences in its reaction toward normal hog, rabbit, and cow sera, and hyperimmune hog serum. The antigen is not present in freshly prepared extract of mesenteric glands, but requires a definite period for development; it is not removed from such an extract by passage through an ordinary porcelain filter but is removed by passage through the F bougie. Finally it gradually disappears from the extract." These investigators state that they are seeking to perfect the preparation of the antigen which they have developed, with a view to rendering it more sensitive.

Our study of *Spirochaeta hyos*,⁴ an organism present in the intestinal ulcers, cecal crypts, and external local lesions of animals suffering from hog cholera, led us to undertake a series of experiments to determine its antigenic value in complement-fixation, a project apparently justified by the results of a rather extensive investigation of this organism.

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¹ Giltner: Tech. Bull. Mich. State Exper. Sta., No. 8, 1911; No. 13, 1912.

² Bull. Missouri State Exper. Sta., 1914, 131, p. 486.

³ Jour. Infect. Dis., 1915, 17, p. 213.

⁴ King and Baeslack: Jour. Infect. Dis., 1913, 12, p. 307. King, Baeslack and Hoffman: *Ibid.*, p. 365. King and Hoffman: *Ibid.*, 13, p. 463. King and Drake: *Ibid.*, 1914, 14, p. 246. King, Drake and Hoffman: *Ztschr. f. Immunitätsf.*, 1914, 22, p. 347. King and Drake: Jour. Infect. Dis., 1915, 16, p. 1.

The complement-fixation test is recognized as one of the most reliable methods of laboratory diagnosis in specific infections such as syphilis, gonorrhea, glanders, contagious abortion, and dourine. The test is also of practical use in the standardization of certain antisera, as antimeningococcic and antigonococcic sera, and in checking up the specificity of pathogenic micro-organisms.

It is unnecessary to discuss in detail the methods which should be followed in routine complement-fixation work, but for the purpose of recording our results clearly and completely, the following explanations are given.

APPARATUS

The tests are carried out in small tubes, 50 mm. in length by 8 mm. in diameter. In addition to the ordinary pipets graduated to 10ths and 100ths, it is a convenience to have small pipets of 0.1-c.c. capacity, made especially for the work from thermometer glass and graduated by mercury into 100 parts, thus affording readings to 0.001 c.c. The solutions used are normal salt solution (0.85 NaCl to 100 c.c. distilled water) and sodium-citrate solution (1% sodium citrate in normal salt solution). Special care is exercised in cleaning tubes, pipets, and other glassware; if chromic-acid cleaning solution is used, the apparatus is very thoroughly rinsed, first in tap water and then in sterile water, before drying and sterilizing, to eliminate inaccuracies due to the presence of foreign matter.

REAGENTS

Sheep Corpuscles.—Fresh sheep blood is collected in sodium-citrate solution. The red cells are secured by repeated centrifugation and at least 4 washings in normal salt solution. Finally the cells are placed in normal salt solution in 1% suspension. This suspension may be kept for several days in the refrigerator.

Amboceptor.—The amboceptor, hemolytic serum, is obtained from a rabbit previously injected intravenously with varying doses of washed sheep corpuscles. The rabbit should receive injections of 4, 6, and 8 c.c. respectively, at intervals of 7 days. Ten days after the last injection, the rabbit is bled and the serum obtained.

Complement.—The complement, normal guinea-pig serum, is obtained each day in fresh condition and it should be clear.

Antigen.—Spirochetes are obtained by centrifugation from a pure liquid culture of *Spirochaeta hyos*. The sediment of pure *Spirochaeta hyos* thus obtained, is washed with normal salt solution, the supernatant liquid removed, and 20 times its volume of absolute alcohol added to the mass of washed spirochetes. The suspension is placed in a mechanical shaker for 24 hours, after which it is incubated at 37 C. for a period of 10 days, being shaken by hand a few times each day during this period. At the end of this time the suspension is diluted with an equal volume of normal salt solution. It is then ready for titration.

In the present work the strain of *Spirochaeta hyos* used in the preparation of antigen was secured from the intestinal ulcers of Hog 112. This animal was infected with a strain of hog-cholera virus received from Dr. Moore and Dr. Birch of Cornell University. The antigen was obtained from pure cultures of *Spirochaeta hyos* grown under oil in ascitic-broth media to which had been added sterile rabbit kidney or testicular tissue.

Serum to be Tested.—The serum to be tested is obtained under aseptic conditions free from red corpuscles and hemolysis. Before use, it is inactivated by heating to 56 C. for 30 minutes in a water bath.

TITRATION OF REAGENTS

Amboceptor.—The serum from the rabbit immunized against sheep corpuscles is inactivated by heating in a water bath at 56 C. for 30 minutes. Dilutions of the serum are then made, from 1:100 to 1:2500, and by titration of these with 0.01 c.c. of complement and 1 c.c. of the 1% suspension of washed sheep cells, that dilution is found in which complete hemolysis of the red cells occurs in 1 hour. This represents the amboceptor unit. Twice this amount is used in the test.

Complement.—The complement, fresh normal guinea-pig serum, is titrated against the amboceptor unit thus obtained, for the purpose of determining any variation in the complementary properties of the fresh guinea-pig sera. This titration is carried out each day before the test. The complementary unit is the smallest amount of complement that will completely hemolyze 1 c.c. of a 1% suspension of sheep cells in the presence of 1 unit of amboceptor. For example, 0.005 c.c. complement after 1 hour at 37.5 C. in the water bath, caused partial or no hemolysis; 0.01 c.c., 0.015 c.c., and 0.02 c.c. gave complete hemolysis while the control remained unhemolyzed. Twice the complementary unit is used in the test.

Antigen.—The antigen must be titrated for the presence of hemolysins. Tubes containing different amounts of antigen, 0.005, 0.01, 0.03, 0.05, and 0.1 c.c., respectively, and one containing no antigen, as control, together with 2 units of complement and 1 c.c. of the sheep-cell suspension, are incubated for 1 hour at 37.5 C. in a water bath. None of the tubes should show hemolysis. If all the tubes show hemolysis, either the complement or the cell suspension, or both, are hemolytic. If there is hemolysis in all the tubes except the control tube, the antigen itself is hemolytic and should be discarded.

The antigen in amounts of 0.005, 0.01, 0.02, 0.05, and 0.1 c.c., is titrated against 2 units of complement, 2 units of amboceptor, and 1 c.c. of sheep-cell suspension for the purpose of detecting the presence of any anticomplementary properties. A control tube containing no antigen is also prepared. If there is complete hemolysis in each case after incubation at 37.5 C. for 1 hour, the absence of anticomplementary properties is demonstrated. If hemolysis occurs in the control tube, and inhibition is present in any of the tubes containing the larger amounts of antigen, the amount of antigen used must be less than that causing any inhibition of hemolysis.

For the purpose of determining its antigenic properties the antigen is titrated against a known positive and a known normal, or negative, serum as illustrated in Table 1.

TABLE 1
THE TITRATION OF ANTIGEN FOR ITS ANTIGENIC PROPERTIES

Tube	Amount of Serum, c.c.	Units of Complement	Amount of Antigen,* c.c.
1	Cholera.....	2	0.005
2		2	0.01
3		2	0.015
4		2	0.02
5		2	0.0
6	Normal.....	2	0.005
7		2	0.01
8		2	0.015
9		2	0.02
10		2	0.0

* The antigen, in the largest amount used, should have previously shown no anticomplementary properties.

The tubes are incubated in a water bath at 37.5 C. for 1 hour. Then to each tube are added 1 c.c. of sheep cells and 2 units of amboceptor and the tubes are again incubated in a water bath at 37.5 C. for 1 hour. At the end of the hour, after the sheep cells are added, there should be complete hemolysis in Tubes 5, 6, 7, 8, 9, and 10, but of the first 4 tubes, those containing sufficient antigen to bind the complement, should show complete inhibition of hemolysis. From this titration the amount of antigen necessary to cause fixation of complement is determined and used as the antigenic unit for the actual test.

THE TEST

Table 2 will illustrate the method of conducting the complement-fixation test.

TABLE 2
COMPLEMENT-FIXATION TEST IN HOG-CHOLERA

Tube	1	2	3	4*
Sera used (c.c.) { 217 (normal)..... 207 (known positive) Lapeer (unknown).... }	0.02	0.04	0.06	0.06
Antigen (c.c.).....	0.01	0.01	0.01	0.0
Complement (c.c.).....	0.03	0.03	0.03	0.03
The tubes were incubated in a water bath for 1 hour at 37.5 C.				
Amboceptor (c.c.).....	0.04	0.04	0.04	0.04
Cells (c.c.).....	1.0	1.0	1.0	1.0
Hemolysis after 40 minutes:				
Serum 217.....	Complete	Complete	Complete	Complete (control)
Serum 207.....	++	—	—	Complete
Serum from Lapeer.....	++	—	—	++

* Since antigen, complement, amboceptor, and cells had been previously tested for anticomplementary properties and hemolysis, the only control of the test was the serum control (Tube 4).

That there was some slight inhibiting action in the case of the Lapeer serum is shown by the failure of Tube 4 to hemolyze completely, but as no hemolysis had taken place in Tubes 2 and 3, a positive reading was given. Of the

tubes containing cholera serum (207), Nos. 2 and 3 showed no hemolysis and therefore positive results were recorded. Hemolysis occurred in all tubes containing Serum 217 (normal); negative results were recorded. It was shown that 0.02 c.c. of serum was insufficient in amount to cause complete complement-fixation—that is, to prevent partial hemolysis.

Results should be read when the action of the controls is complete. If the test shows complete hemolysis in the tubes (see Tubes 1, 2, and 3), it is evident that there has been no fixation of complement by the serum; therefore, the serum is negative (—). If there has been no hemolysis, the complement is bound and the serum is positive (++++). If only a slight degree of hemolysis has taken place, the result is recorded as triple plus (+++). When only about one-half of the cells have hemolyzed, the reading is given double plus (++), while if there are only a few cells left unhemolyzed but still easily seen, a reading of one plus (+) is made. A one plus (+) is interpreted as doubtful; double plus (++), triple (+++), and four plus (++++), as positive.

The readings should be checked up after the tubes have been allowed to stand for several hours; the tubes containing known normal serum should show complete hemolysis, while those representing positive, or cholera, serum, should remain unhemolyzed. The action frequently occurs in less than 1 hour after the cells are added, and in such cases rapid hemolysis may be partially checked by placing the tubes in an incubator or at room temperature instead of in the water bath.

Known positive and negative sera must be subjected to test with the unknown sera to insure proper titration of all reagents. It is obvious that the amounts of sera used should be varied, as, for example, in the test described, in which the smaller amount of serum was found insufficient to cause complete complement-fixation. The amounts of sera used should not be too large on account of the inhibition of hemolysis which might result. This is controlled by Tube 4. Occasionally a serum is found which possesses inhibitory properties. Such a serum must be titrated carefully to determine the amount in which inhibition is negligible.

THE STRAINS OF VIRUS UTILIZED

Five different strains of hog-cholera virus have been used in conducting these experimental complement-fixation tests. Strain 1 (N. Y.) was received from Dr. Moore and Dr. Birch, of Cornell University. Strain 2 (from Dr. Hauk, of East St. Louis) represented a stock strain built up by mixing together all the strains of virus obtainable. Some of the original strains incorporated in this were secured from field cases, some from the government laboratories at Ames, Iowa, and some from serum-manufacturing laboratories. Dr. Hadley and Dr. Beach, of the University of Wisconsin, furnished Strain 3 (Wisconsin). Strain 4 was secured on October 21 from a cholera-infected herd of hogs at Grosse Isle, Mich. During the week of October 25, hog cholera appeared on the farm of the Michigan state school for feeble-minded children, at Lapeer. One test was conducted with a specimen of serum obtained during this outbreak (Strain 5, Lapeer).

Serum was tested from one animal, Hog 63, infected with Strain 6 (Eloise). Hog 63 had received impure cultures of *Spirochaeta hyos* isolated from the intestinal ulcers in pigs that had received virus from an outbreak of hog cholera on the farm of the Wayne county hospital, Eloise, Mich.

The following hogs, infected with the different strains of virus, were used in the complement-fixation tests:

Strain 1 New York	Strain 2 St. Louis	Strain 3 Wisconsin	Strain 4 Grosse Isle	Strain 5 Lapeer	Strain 6 Eloise	Unknown Strains
77	187	188	206	Strain from case in field	63	192
160	161	203	223			189
186	202	217	227			220
106	141	205				
87	207	216				
204	224	218				
208	229	222				
221		214				
230		228				
		215				

SUMMARY OF GENERAL DATA

With antigen prepared from pure cultures of *Spirochaeta hyos* there have been conducted 115 complement-fixation tests. Of these, 22 were with normal hog sera from 10 different animals, 1 with serum from an animal which exhibited a reaction only, following inoculation with virus, 6 with sera from 2 convalescent or naturally immune swine, 84 with sera from 34 animals suffering from hog cholera (4 of which had been used as normals), and 1 test each with 2 different lots of hyper-immune serum. Table 3 shows the results obtained.

TABLE 3
COMPLEMENT-FIXATION TESTS WITH ANTIGEN FROM PURE CULTURES OF SPIROCHAETA HYOS

Test	Date of Test	Animal	Date of Collection of Serum	Clinical Condition of Animal	Number of Days After Inoculation	Result of Complement Fixation Test	Remarks
1	10/ 5	Normal A	10/ 5	Normal	..	—	
2	10/ 5	77	3/23	Hog cholera	10	++++	Autopsy on 10th day
3	10/ 7	77	3/23	Hog cholera	10	+++	
4	10/ 5	160	8/18	Hog cholera	9	++	Autopsy on 9th day
5	10/22	160	8/18	Hog cholera	9	++	
6	10/ 7	Normal B	10/ 7	Normal	..	—	
7	10/ 8	Normal B	10/ 7	Normal	..	—	
8	10/ 8	186	10/ 8	Hog cholera	10	—	Error in technic. See Test 9
9	10/13	186	10/ 8	Hog cholera	10	++++	Autopsy on 16th day
10	10/ 8	187	10/ 4	Hog cholera	17	++++	Autopsy on 18th day
11	10/ 8	188	10/ 3	Hog cholera	12	+++	Autopsy on 12th day
12	10/13	Normal C	10/13	Normal	..	—	
13	10/26	Normal C	10/13	Normal	..	—	
14	10/13	106	5/ 6	Hog cholera	10	++++	Autopsy on 10th day
15	10/13	161	8/23	Hog cholera	13	++++	
16	10/22	161	8/23	Hog cholera	..	++++	
17	10/26	161	8/23	Hog cholera	..	++++	
18	11/ 4	161	8/23	Hog cholera	..	++	
19	10/21	Normal D	10/20	Normal	..	—	
20	10/22	Normal D	10/20	Normal	..	—	

TABLE 3—Continued

COMPLEMENT-FIXATION TESTS WITH ANTIGEN FROM PURE CULTURES OF SPIROCHAETA HYOS

Test	Date of Test	Animal	Date of Collection of Serum	Clinical Condition of Animal	Number of Days After Inoculation	Result of Complement-Fixation Test	Remarks
21	10/21	192	10/20	Hog cholera	13	—	Error in technic. See Test 23
22	10/22	192	10/20	Hog cholera	13	±	Error in technic. See Test 23
23	10/26	192	10/20	Hog cholera	13	++++	Autopsy on 19th day
24	10/21	Hyperimmune serum	+++	Received from Michigan exper. station
25	10/21	87	4/15	Hog cholera	10	++++	Autopsy on 10th day
26	10/21	63	1/29	Hog cholera	16	++++	
27	10/29	63	1/29	Hog cholera	16	++++	Autopsy on 16th day
28	10/22	203	10/22	Hog cholera	10	+	See Test 29
29	10/26	203	10/22	Hog cholera	10	++++	Died on 17th day
30	10/22	202	10/22	Hog cholera	10	+	Died on 31st day
31	10/22	Grosse Isle	10/21	Hog cholera	..	+	Secured in field from moribund animal. See Test 32
32	10/26	Grosse Isle	10/21	Hog cholera	..	+++	Secured in field from moribund animal
33	10/29	217	10/29	Normal	..	—	
34	11/ 2	217	10/29	Normal	..	—	
35	11/12	217	11/12	Hog cholera	8	++	Autopsy on 13th day
36	10/29	204	10/27	Symptoms, Temp. 106 F. No symptoms	9	++	Animal had reaction only
37	11/ 2	204	11/ 1	No symptoms	14	+	Became normal on 13th day
38	11/11	204	11/10	Normal immune	23	—	Exposed with Hogs 208 and 221
39	11/29	204	11/22	Normal immune	34	—	
40	12/ 2	204	11/30	Normal immune	42	—	
41	10/29	Hyperimmune serum	+++	Received from Dr. Huff, Sioux City, Iowa
42	10/29	141	9/11	Hog cholera	14	++++	Autopsy on 14th day
43	11/ 2	141	9/11	Hog cholera	..	++++	
44	11/ 2	205	11/ 1	Hog cholera	4	++	Temp. 105. Clinical symptoms 5th day
45	11/10	205	11/ 9	Hog cholera	12	+++	
46	11/11	205	11/ 9	Hog cholera	12	++	
47	11/19	205	11/17	Hog cholera	20	++++	Found dead on 23rd day
48	11/ 2	206	11/ 1	Natural immune	4	—	
49	11/ 2	207	11/ 1	Hog cholera	4	+++	Temp. 105.8. Clinical symptoms. Found dead on 19th day
50	11/ 2	Lapeer serum	10/30	Hog cholera	..	++++	Natural exposure in field
51	11/ 9	216	11/ 3	Normal	..	—	
52	11/10	216	11/ 3	Normal	..	—	
53	11/12	216	11/12	Hog cholera	8	+++	Autopsy on 13th day
54	11/ 4	220	11/ 3	Hog cholera	?	++++	Accidental exposure
55	11/11	220	11/ 3	Hog cholera	?	+++	Found dead on 13th day
56	11/ 4	208	11/ 3	Hog cholera	6	++++	Symptoms
57	11/10	208	11/ 9	Hog cholera	12	++	
58	11/15	208	11/10	Hog cholera	13	++++	
59	11/15	208	11/ 9	Hog cholera	12	++++	
60	11/16	208	11/ 9	Hog cholera	12	++++	
61	11/29	208	11/24	Hog cholera	27	+++	Moribund
62	11/ 4	189	11/ 4	Hog cholera	?	+++	Accidental exposure. Found dead 11/12
63	11/10	Normal E	11/ 9	Normal	..	—	
64	11/11	Normal E	11/ 9	Normal	..	—	
65	11/18	Normal E	11/ 9	Normal	..	—	

SPIROCHAETA-HYOS ANTIGEN IN COMPLEMENT-FIXATION 53

TABLE 3—Continued

COMPLEMENT-FIXATION TESTS WITH ANTIGEN FROM PURE CULTURES OF SPIROCHAETA HYOS

Test	Date of Test	Animal	Date of Collection of Serum	Clinical Condition of Animal	Number of Days After Inoculation	Result of Complement-Fixation Test	Remarks
66	11/11	218	11/10	Hog cholera	6	+	Autopsy on 13th day
67	11/12	221	11/11	Normal	..	—	
68	11/12	221	11/12	Hog cholera	1	—	No symptoms, no fever
69	11/15	221	11/12	Hog cholera	1	—	No symptoms, no fever
70	11/29	221	11/12	Hog cholera	1	—	
71	11/15	221	11/13	Hog cholera	2	—	
72	11/16	221	11/13	Hog cholera	2	—	
73	12/ 1	221	11/13	Hog cholera	2	—	No symptoms, no fever
74	11/16	221	11/14	Hog cholera	3	—	
75	11/16	221	11/15	Hog cholera	4	++	
76	11/18	221	11/15	Hog cholera	4	+++	
77	11/16	221	11/16	Hog cholera	5	+	Slightly inactive, no fever
78	11/19	221	11/16	Hog cholera	5	+++	Temp. 104.2
79	11/18	221	11/17	Hog cholera	6	+++	Error in technic. See Test 81
80	11/18	221	11/18	Hog cholera	7	—	Temp. 106. Symptoms
81	11/19	221	11/18	Hog cholera	7	++++	
82	11/19	221	11/19	Hog cholera	8	++++	
83	11/29	221	11/22	Hog cholera	11	+++	
84	12/ 2	221	11/24	Hog cholera	13	++++	
85	12/ 7	221	11/29	Hog cholera	18	++++	Animal died on 20th day
86	11/15	222	11/11	Normal	..	—	
87	11/12	222	11/12	Hog cholera	1	—	
88	11/15	222	11/12	Hog cholera	1	—	
89	11/15	222	11/13	Hog cholera	2	—	
90	11/16	222	11/14	Hog cholera	3	+++	Temp. 106.2. No clinical symptoms
91	11/16	222	11/15	Hog cholera	4	+++	
92	11/16	222	11/16	Hog cholera	5	+++	Clinical symptoms pronounced
93	11/18	222	11/17	Hog cholera	6	+++	
94	11/18	222	11/18	Hog cholera	7	+	See Test 95
95	11/19	222	11/18	Hog cholera	7	++++	
96	11/19	222	11/19	Hog cholera	8	++++	Found dead on 10th day
97	11/15	Normal F	11/15	Normal	..	—	
98	11/19	Normal F	11/15	Normal	..	—	
99	11/18	223	11/17	Hog cholera	6	—	Field virus, Grosse Isle. Temp. 105.4. Clinical symptoms
100	11/29	223	11/22	Hog cholera	11	+++	Found dead on 21st day
101	11/29	214	11/22	Hog cholera	11	+++	
102	12/ 1	214	11/22	Hog cholera	11	+++	
103	12/ 2	214	11/22	Hog cholera	11	+++	Found dead on 18th day
104	11/29	215	11/19	Chronic hog cholera	8	—	No symptoms. Temp. 104.2
105	12/ 2	215	11/29	Chronic hog cholera	18	+++	Marked symptoms
106	11/29	224	11/19	Hog cholera	8	+++	Autopsy on 25th day
107	12/ 7	227	12/ 2	Hog cholera	5	+	Temp. 104. Slight symptoms
108	12/ 7	227	12/ 4	Hog cholera	7	+++	Temp. 106. Marked symptoms
109	12/ 7	228	12/ 2	Hog cholera	5	+	Temp. 106. Symptoms
110	12/ 7	228	12/ 4	Hog cholera	7	++++	Temp. 107. Symptoms
111	12/ 7	Normal G	12/ 6	Normal	..	—	
112	12/ 7	229	12/ 2	Hog cholera	5	+	No symptoms. Temp. 103.6
113	12/ 7	229	12/ 4	Hog cholera	7	++	No symptoms. Temp. 104.8
114	12/ 7	230	12/ 2	Hog cholera	5	+	No symptoms. Temp. 104
115	12/ 7	230	12/ 4	Hog cholera	7	+++	No symptoms. Temp. 105.4

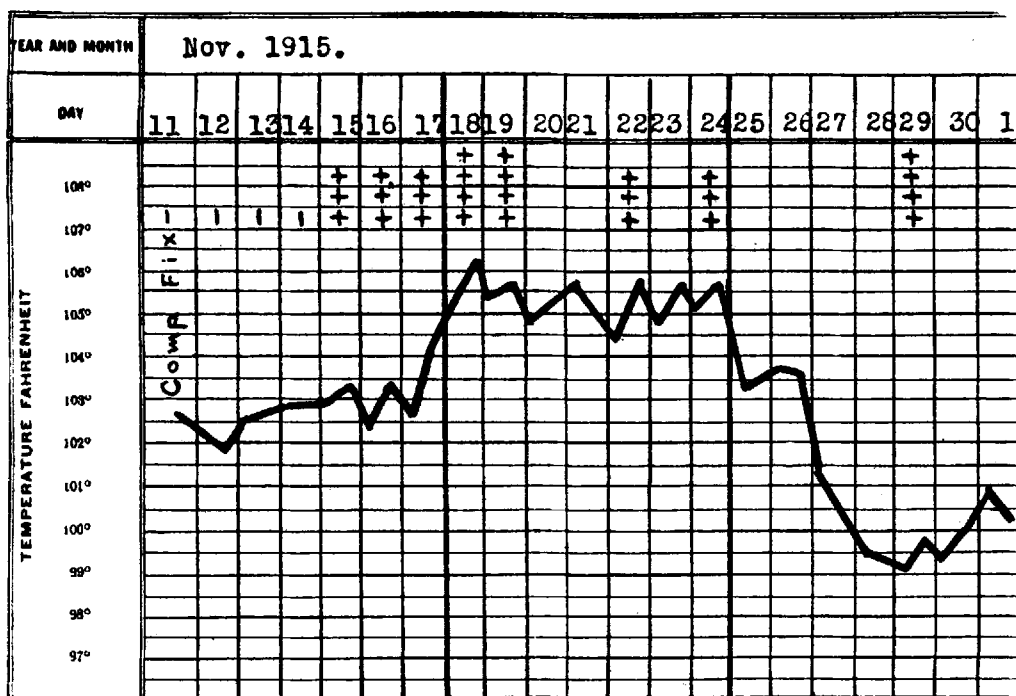


Chart 1. Clinical chart of Hog 221, showing also the time of the appearance of complement-fixation. November 11, intramuscular injection of 2 c.c. of Virus 208, Strain 1 (N. Y.). November 16, slight symptoms. November 18, marked symptoms. November 22, acute hog cholera. December 2, found dead. Examined.

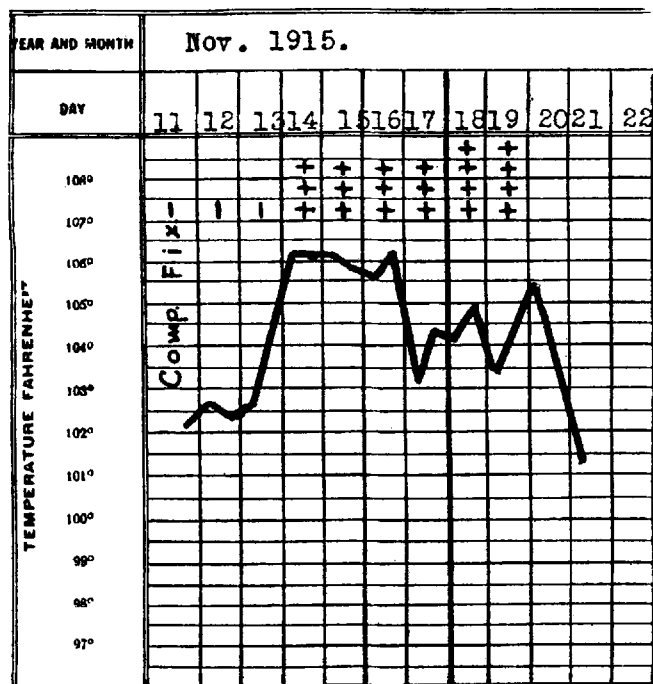


Chart 2. Clinical chart of Hog 222, showing also the time of the appearance of complement-fixation. November 11, intramuscular injection of 2 c.c. of Virus 205, Strain 3 (Wis.). November 15, slight symptoms. November 16, marked symptoms. November 17, acute hog cholera. November 22, found dead. Examined.

These results may be summarized as follows: (1) Hemolysis (—) occurred in all cases in which normal hog sera were subjected to complement-fixation test. (2) Complement-fixation (+) resulted in all tests with sera from cholera hogs, except in Nos. 30 and 66

THE TIME OF THE APPEARANCE OF A POSITIVE REACTION

In order to determine the number of days after inoculation before complement-fixation appears, daily examinations were made of the sera of two experimentally infected animals (221 and 222). The results obtained are shown in Charts 1 and 2:

With the sera of Hogs 222 and 221, positive serum reactions occurred in 3 and 4 days, respectively. These results corresponded approximately with the apparent periods of incubation, variation in resistance, and types of the disease present in these animals. Hog 222 exhibited a temperature of 106.2 on the morning of the 3rd day, clinical symptoms on the 4th day, and died on the 10th day. Hog 221, the serum of which gave a positive reaction one day later than that of Hog 222, did not show clinical symptoms until the 5th day, or rise of temperature until the 6th day, and lived until the 20th day.

Additional data bearing on this point are presented in Table 4.

TABLE 4
THE TIME OF THE APPEARANCE OF COMPLEMENT-FIXATION

Hog	Incubation Period According to Temperature and Clinical Conditions	Duration of Disease in Days	Type of Disease	Complement-Fixation Test		Results of Subsequent Complement-Fixation Tests
				Day After Inoculation	Result	
205	4 days	23	Subacute	4th	++	12th day, +++; 20th day, ++++
207	4 days	19	Subacute	4th	+++	
227	4 days	..	Acute	5th	+	7th day, +++
228	4 days	..	Acute	5th	+	7th day, ++++
229	7 days	..	Subacute	5th	+	7th day, ++
230	7 days	..	Subacute	5th	+	7th day, +++
208	5 days	27	Chronic	6th	++++	12th and 13th days, ++++; 27th day, +++
218	4 days	13	Acute	6th	+	
223	6 days	21	Subacute	6th	—	11th day, +++
215	9 days	31	Chronic	8th	—	18th day, +++

The results of serum tests applied before symptoms appear or early in the course of the disease, indicate that complement-fixation is coincident with clinical symptoms, and that the time of its appearance depends on the virulence of the infecting material and the individual resistance of the animal.

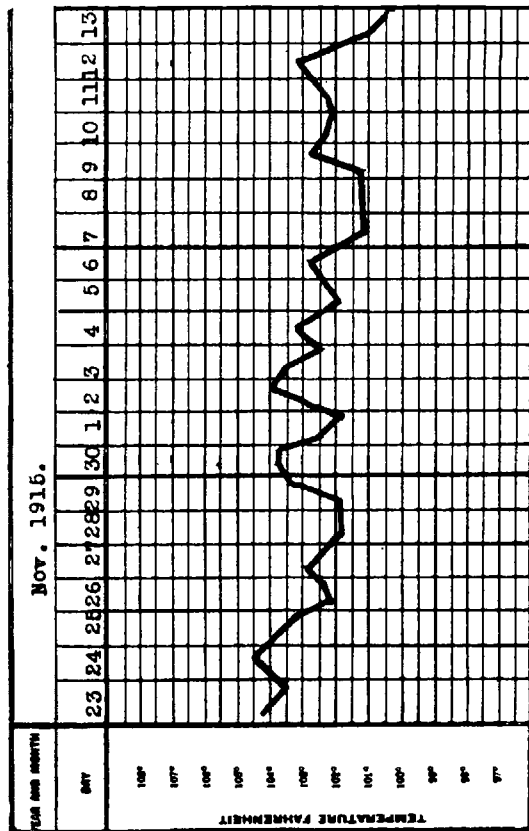
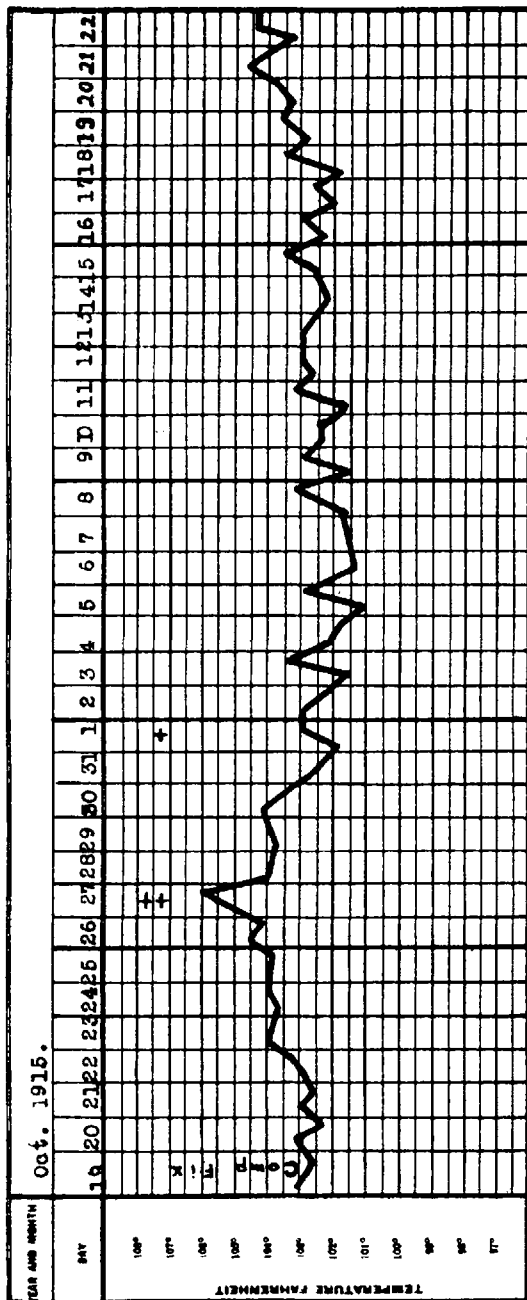


Chart 3. Clinical chart of Hog 204, showing also the results of tests for the duration of complement-binding substances in the blood of Hog 204. October 19, intramuscular injection of 3 c.c. of Virus 160 and of Virus 186. October 25, seemed well. October 27, symptomatic. October 31, seemed well. November 3, normal. November 10, normal. November 20, placed in box beside radiator inside. November 24, placed in box outside and bedding kept water-soaked. December 1, cough. December 6, inactive. December 8, seemed normal. December 10, started on salt treatment. December 12, very ill. December 13, moribund.

DURATION OF COMPLEMENT-BINDING SUBSTANCES IN BLOOD OF
IMMUNE HOGS

During the course of these experiments one naturally immune hog was found. This animal, Hog 206, was inoculated on October 28 with 2 c.c. of serum from a typical case of hog cholera in a natural field outbreak at Grosse Isle, Mich. On the 4th day after inoculation, serum from Hog 206 failed to fix complement. No symptoms of cholera appeared altho the Grosse-Isle serum was virulent for other hogs. Hog 206 was subjected to natural exposure without results, and later was used for other purposes. The serum from this animal continued to be negative in complement-fixation tests. Serum from Hog 204 was also submitted to several tests (see Chart 3).

The results of these tests on the sera of Hogs 206 and 204 indicate that complement-binding substances cease to exist in the blood of hogs when immunity against hog cholera becomes fully established.

CONTROL ANTIGENS

In order that there might be some method of control in this work with pure *Spirochaeta-hyos* antigen, the following control antigens, prepared according to the method used in making the original spirochete antigen, were tested.

1. *B. cholera-suis* antigen from a pure culture of *B. cholera-suis* received several years ago from Theobald Smith.
2. *B. Voldagsen* antigen from a pure culture of *B. Voldagsen* received from Dr. Haendel, Königliches Hygienisches Institut, Germany, April, 1914.
3. *B. typhi-suis* (Glaesser) antigen from a pure culture of *B. typhi-suis*, also received from Dr. Haendel.
4. *Spirochaeta-hyos* Antigen 2 from a pure liquid culture from Hog 112 (New York strain).

These antigens were all prepared at the same time and tested with results as given in Table 5.

In these comparative tests with the control antigens, the maximal amounts which would not cause anticomplementary reactions were used. The results show that antigens prepared from pure cultures of *B. cholera-suis*, *B. typhi-suis*, and *B. Voldagsen*, as compared with two lots of pure *Spirochaeta-hyos* antigen, contain no specific complement-binding properties for hog-cholera serum.

A comparison of *Spirochaeta-hyos* Antigens 1 and 2, which were 3 month and 1 month old, respectively, showed the more recently prepared material to be slightly more active.

TABLE 5
RESULTS OF COMPLEMENT-FIXATION TESTS WITH CONTROL ANTIGENS

Date of Tests	Serum Tested	Amount of Serum c.c.	Antigen	Amounts of Antigen Used, c.c.	Results
11/24	Normal B	0.04	B. Voldagsen	0.005, 0.0075, 0.01	—
11/24	Normal B	0.06	B. Voldagsen	0.005, 0.0075, 0.01	—
11/24	Normal B	0.04	Sp. hyos 2	0.005, 0.0075, 0.01	—
11/24	Normal B	0.06	Sp. hyos 2	0.005, 0.0075, 0.01	—
11/24	Cholera 187	0.04	B. Voldagsen	0.005, 0.0075, 0.01	—
11/24	Cholera 187	0.06	B. Voldagsen	0.005, 0.0075, 0.01	—
11/24	Cholera 187	0.04	Sp. hyos 2	0.005, 0.0075, 0.01	+++
11/24	Cholera 187	0.06	Sp. hyos 2	0.005, 0.0075, 0.01	++++
11/26	Normal B	0.01, 0.02, 0.03, 0.04	B. typhi-suis	0.005, 0.01	—
11/26	Cholera 187	0.01, 0.02, 0.03, 0.04	B. typhi-suis	0.005, 0.01	—
11/26	Normal B	0.01, 0.02, 0.03, 0.04	B. cholera-suis	0.005, 0.01	—
11/26	Cholera 187	0.01, 0.02, 0.03, 0.04	B. cholera-suis	0.005, 0.01	—
11/26	Normal B	0.01, 0.02, 0.03, 0.04	B. Voldagsen	0.005, 0.01	—
11/26	Cholera 187	0.01, 0.02, 0.03, 0.04	B. Voldagsen	0.005, 0.01	—
11/26	Normal B	0.01, 0.02, 0.03, 0.04	Sp. hyos 2	0.005, 0.01	—
11/26	Cholera 187	0.01, 0.02, 0.03, 0.04	Sp. hyos 2	0.005	+++
11/26	Cholera 187	0.01, 0.02, 0.03, 0.04	Sp. hyos 2	0.01	++++
11/26	Cholera 187	0.01, 0.02, 0.03, 0.04	Sp. hyos 1	0.005	++++
11/26	Cholera 187	0.01, 0.02, 0.03, 0.04	Sp. hyos 1	0.01	++++
11/26	Normal B	0.01, 0.02, 0.03, 0.04	Sp. hyos 1	0.005	—
11/26	Normal B	0.01, 0.02, 0.03, 0.04	Sp. hyos 1	0.01	—
12/ 2	Normal 204	0.06	Sp. hyos 2	0.01	—
12/ 2	Normal 204	0.06	B. Voldagsen	0.01	—
12/ 2	Normal 204	0.06	B. typhi-suis	0.01	—
12/ 2	Normal 204	0.06	B. cholera-suis	0.01	—
12/ 2	Normal 204	0.06	Sp. hyos 1	0.01	—
12/ 2	Cholera 221	0.06	Sp. hyos 2	0.01	+++
12/ 2	Cholera 221	0.06	B. Voldagsen	0.01	—
12/ 2	Cholera 221	0.06	B. typhi-suis	0.01	—
12/ 2	Cholera 221	0.06	B. cholera-suis	0.01	—
12/ 2	Cholera 221	0.06	Sp. hyos 1	0.01	+++
12/15	Cholera 215	0.06	Sp. hyos 2	0.02	+++
12/15	Cholera 215	0.06	Sp. hyos 1	0.02	++
12/15	Cholera 215	0.06	B. Voldagsen	0.02	—
12/15	Cholera 215	0.06	B. typhi-suis	0.02	—
12/15	Cholera 223	0.06	B. cholera-suis	0.02	—
12/15	Cholera 223	0.06	Sp. hyos 2	0.02	++
12/15	Cholera 223	0.06	B. Voldagsen	0.02	—
12/15	Cholera 223	0.06	B. typhi-suis	0.02	—
12/15	Cholera 223	0.06	B. cholera-suis	0.02	—
12/ 3	Normal 204	0.04	Sp. hyos 2	0.02	—
12/ 3	Normal 204	0.04	B. typhi-suis	0.02	—
12/ 3	Cholera 227	0.04	Sp. hyos 2	0.02	+++
12/ 3	Cholera 227	0.04	B. typhi-suis	0.02	—
12/ 3	Early cholera 228	0.04	Sp. hyos 2	0.02	+
12/ 3	Early cholera 228	0.04	B. typhi-suis	0.02	—
12/ 3	Early cholera 229	0.04	Sp. hyos 2	0.02	++
12/ 3	Early cholera 229	0.04	B. typhi-suis	0.02	—
12/ 3	Early cholera 230	0.04	Sp. hyos 2	0.02	+++
12/ 3	Early cholera 230	0.04	B. typhi-suis	0.02	—

CONTROL COMPLEMENT-FIXATION TESTS WITH SERA OF HOGS SUFFERING FROM DISEASES OTHER THAN HOG CHOLERA

In considering the possible specificity of the Spirocheta-hyos antigen in complement-fixation tests with hog-cholera serum, it appeared necessary to determine the results of the application of the test to sera obtained from hogs suffering from disease processes other than that of hog cholera. The committee on diseases, of the American Veterinary Medical Association, in August, 1915, reported as follows concerning the differential diagnosis of hog cholera:

Among the diseases or disease conditions that must be differentiated from hog cholera, are parasitism, a form of infectious enteritis, that condition which the U. S. Bureau of Animal Industry calls Salmonellosis and is supposed to be due to the *Bacillus suispestifer*, the so-called swine plague, pneumonia, verminous pneumonia, brine poisoning, acute pericarditis, shoat typhoid, enteritis and poisoning from spoiled foods, soap powders and irritating stock powders, swine erysipelas (which so far as we know does not exist in this country) septicemia, malignant edema, necrotic laryngitis, anthrax, heat stroke, lightning stroke, or sudden death from any cause, and a number of acute febrile conditions, that we have met with in pigs, but so far have been unable to classify.

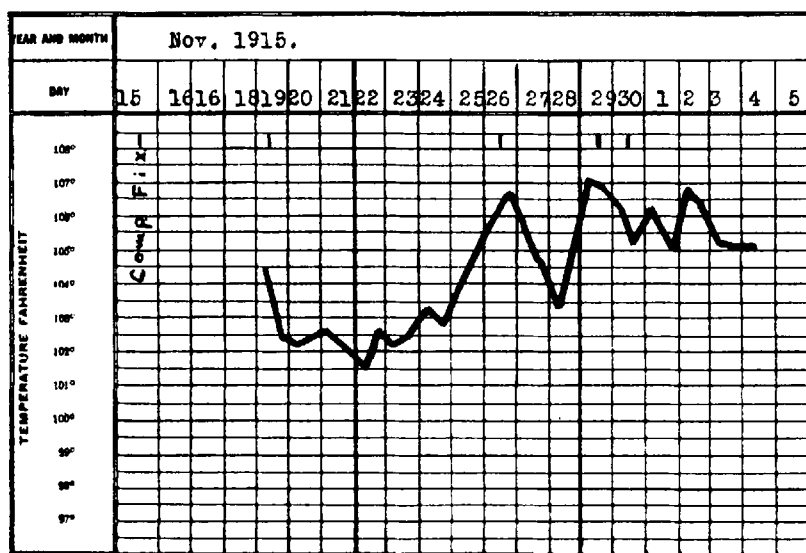


Chart 4. Clinical chart of Hog 225, which had been experimentally infected with *Staphylococcus aureus*. Results of complement-fixation tests are shown at the top of the chart. November 18, intramuscular and subcutaneous injections of 10 c.c. of a mixed broth culture of *Staph. albus*, *Staph. aureus*, and *streptococcus*. November 19, swelling at sites of injection. November 20, intramuscular injection of 10 c.c. mixed staphylococcus cultures Nos. 10, 15, 16 from hogs, and pyocyanus (Ward). November 26, animal dull. November 29, ill. December 4, killed and examined.

In this investigation some of the foregoing pathologic conditions have been experimentally produced:

Septicemia.—Hog 225 (see Chart 4) exhibited typical clinical symptoms of septicemia and bacteremia. Fifteen days after the first inoculation the animal, which had been kept under carefully isolated conditions during the experiment, was killed and examined. The animal was not emaciated. There were swelling and induration at the points of inoculation. Lymphatic glands enlarged and hemorrhagic. Lungs contained a few small hemorrhagic points and one or two small areas of congestion. Heart, spleen, and liver normal. Kidneys slightly congested and covered with a few petechiae. Ental surface of bladder normal. Intestinal tract normal except for the presence of *Ascaris suum* and a slight inflammation of the mucous membrane of the large intestine.

⁵ Jour. Am. Vet. Med. Assn., 1915, 48, p. 221.

Flask broth cultures, made from the heart blood under aseptic conditions, after 24 hours incubation yielded pure colonies of *Staphylococcus aureus* in agar transfers.

B. Cholera-Suis Infection.—Hog 231 (see Chart 5) showed the following: Lymphatic glands enlarged but only slightly congested. Both lungs filled with numerous small hemorrhagic areas. Heart and liver normal. Spleen normal in size, but congested in areas and soft in consistency. Kidneys congested and from 1 to 5 petechiae present. Mucosa of large intestine normal except for a few areas of ecchymosis. Ental surface of bladder normal. *B. cholera-suis* recovered in pure culture from the heart blood.

Anthrax.—On November 20, Hog 226 was injected intramuscularly with 2 c.c. of a 24-hour broth culture of *B. anthracis*. On November 22, as this animal showed symptoms of illness and a temperature of 104.4, a specimen of serum was collected and submitted to complement-fixation test, with negative

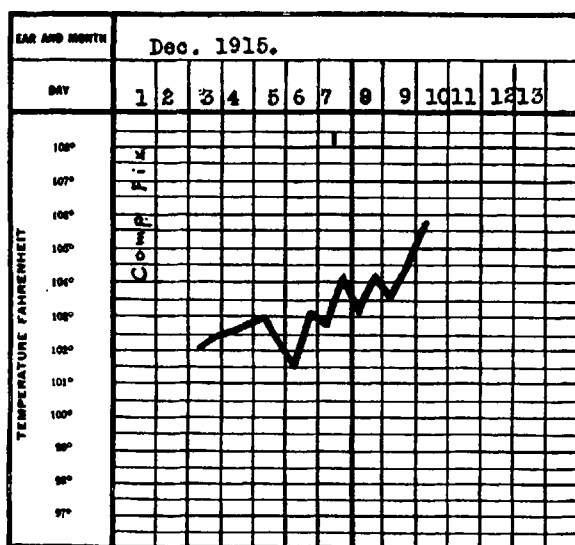


Chart 5. Clinical chart of Hog 231, which had been experimentally infected with *B. cholera-suis*. Results of complement-fixation tests shown at the top of the chart. December 1, intramuscular injection of 11 c.c. of a culture broth of *B. suis* 052 (Theobald Smith). Given 5 c.c. of same cultures orally. December 7, ill. December 9, anorexia. December 10, very ill; killed and examined.

results. On November 24, the condition of Hog 226 was normal and subsequent injection with massive doses of *B. anthracis* demonstrated that the animal had acquired active immunity.

Hog 233 was injected with 20 c.c. of a virulent broth culture of *B. anthracis* on December 9. The animal, moribund on December 12, was killed; specimens of heart blood were secured for serum tests and cultures, and an autopsy made. Edematous swelling at point of inoculation. Enlarged engorged spleen. Kidneys congested. Lymphatic glands enlarged and hemorrhagic. Cultures from heart blood yielded pure *B. anthracis*. Complement-fixation tests of the serum from this animal with *Spirochaeta-hyos* antigen resulted negatively.

Ghon-Sachs-Bacillus Infection.—Ten cubic centimeters of a deep glucose-agar culture of the Ghon-Sachs bacillus⁶ (original furnished by Dr. K. F. Meyer) were injected intramuscularly into Hog 232 on December 9. The following day the site of injection was surrounded by a large tender edema, the animal was inactive, and the temperature had risen to 104.6. A specimen of serum was secured from Hog 232 on December 10. The complement-fixation test resulted negatively. The 4th day after inoculation the swelling decreased, temperature fell to 102, and the animal resumed normal condition.

Brine or Salt Poisoning and Pneumonia.—Hog 204, immune to hog cholera (see Chart 3), was utilized for experimental brine poisoning. It will be noted in the clinical chart for this animal that an attempt was made to produce pneumonia. From November 20 to 24, Hog 204 was kept beside a warm radiator, after which it was exposed to cold and dampness. During this period the animal developed a cough, irregular temperature, and chills. Specimens of serum collected on November 22 and November 30 failed to show complement-fixation with the spirochete antigen.

From December 10 to 12, Hog 204 was given salt. On December 12 the animal showed pronounced symptoms of brine poisoning and on December 14 death occurred. A complement-fixation test with the serum secured December 12 resulted negatively. The findings at autopsy were as follows: Animal emaciated. Lungs congested, showing also large areas of gray hepatization. Lymphatic glands enlarged but not hemorrhagic. Pericarditis present. Heart enlarged. Liver mottled, engorged with blood, and enlarged. Spleen and kidneys normal in appearance. Intestinal mucosa congested.

These results show that antigen prepared from a pure culture of *Spirochaeta hyos* possesses no complement-binding properties when brought into contact with the sera of hogs suffering from septicemia (*Staph. aureus*), from infection with *B. cholera-suis*, *B. anthracis*, or the Ghon-Sachs bacillus, from brine poisoning, or from pneumonia by natural exposure.

DISCUSSION

In reviewing the method used in these complement-fixation tests, and in attempting to emphasize the importance of careful technic and proper controls, we wish to quote the following from a recent article by Watson:⁷

The successful practice of the complement-fixation test depends mainly upon the preparation and use of powerful reagents, their specificity and the accurate determination of their relative values, the fixing of standard doses wherever possible, and a constant, uniform technique and method of procedure.

Close familiarity with the activity of the reagents is essential for the best results.

Stock reagents should be prepared in quantities calculated to meet all requirements for as long a time as the activity of the reagents remains practically constant. Thus: sufficient hemolytic serum for six months' work;

⁶ Meyer: Jour. Infect. Dis., 1915, 17, p. 458.

⁷ Parasitology, 1915, 8, p. 156.

antigen to suffice for one month's work; fresh red cell suspension once a week; fresh complement daily or on alternate days, or as needed. It is advisable to use the blood of two sheep for sensitizing rabbits and to use the red cells of the same sheep for the hemolytic system.

The following points of extreme importance will bear repetition:

1. The amount of red cells in suspension must be very accurately measured and the standard amount never varied.
2. The use of the least possible amount of complement which with two units of hemolytic serum causes complete hemolysis of red cells.
3. The use of twice the amount of antigen which with a dourine antibody unit is necessary to fix the complement, provided the same amount of antigen alone has no inhibitory action.
4. Careful control of the inactivation of suspected sera by known positive and known negative sera.
5. Control of the diagnostic tests by a series of known positive sera, each having an antibody unit of different value, high to low.

This quotation applies equally well to the subject of complement-fixation in hog cholera except for the antibody unit of the serum, the determination of which has not been attempted.

CONCLUSIONS

Antigen prepared from *Spirochaeta hyos* grown in pure culture possesses well-marked specific complement-binding properties.

This antigen, when brought into contact with the sera of experimentally infected cholera hogs, produces initial complement-fixation at a period coincident with completion of the incubation period as observed in clinical conditions and thermal reactions. The specific properties of the antigen are shown to be present until death of the animal, or until active immunity is fully established.

The sera of normal hogs and those experimentally infected with *B. cholera-suis*, the Ghon-Sachs bacillus, *B. anthracis*, *Staph. aureus*, and also, the serum of one hog which was the subject of pneumonia from natural exposure, and which died from acute brine poisoning, all reacted negatively when tested for complement-fixation with *Spirochaeta-hyos* antigen.

We believe that, with the observance of proper technic, the results recorded here can be duplicated without difficulty and that the method may be used to practical advantage as a reliable accurate means of laboratory diagnosis of hog cholera. Furthermore, the results of these experiments support our former conclusions that *Spirochaeta hyos* merits serious consideration as an organism possessing specific pathogenic properties in relation to hog cholera.