STUDIES ON COMPLEMENT FIXATION.

I. The Rate of Fixation of Complement at Different Temperatures.

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INTRODUCTION.

A more complete understanding of the complement fixation phenomenon will perhaps ultimately be achieved by the employment of purified proteins as antigens. The mass of accumulated studies of this phenomenon with organ extracts as antigens may be said to apply largely to the Wassermann test. For, it is questionable whether a principle derived with a non-specific organ extract antigen can apply in all cases to specific antigen-antibody complexes. These Wassermann studies might indeed explain the limited application of the complement fixation test to the diagnosis of diseases other than syphilis and to the identification of unknown organisms.

The antigen, however, is not the only element in which the Wassermann test varies from specific complement fixation tests. Recent studies reported from this laboratory (1) indicate that the behavior of the so called syphilitic complement-fixing antibody towards heat is markedly different from the behavior towards this agent of the complement-fixing antibody found in rabbits after immunization with purified proteins. The former is completely destroyed at temperatures ranging between 62° and 65°C., while the thermal destructive point of the latter is in the neighborhood of 80°C.

The complement fixation reaction, furthermore, is so complex, that the reduction of even one of its ingredients to the simplest elements ought to help in its interpretation. And although chemically the protein molecule is a huge structure indeed, biologically it stands out

as the simplest possible element capable of producing complement-fixing antibodies (2).

These considerations led us to undertake a series of investigations of the complement fixation phenomenon with purified proteins as antigens. These studies are still being continued and are being extended also to organ extracts with syphilitic sera as well as bacterial antigens with their specific antisera with a view of finding whether the principles developed by means of protein antigens are applicable also to practical tests.

The factors governing the fixation of complement were chosen as the subject of the first paper of this series, because of their fundamental importance in all complement fixation studies. Investigations on the effects of temperature and time on the fixation of complement have heretofore been restricted largely to the Wassermann test. The literature covering this field will be presented in a forthcoming paper in which the rate of fixation of complement in the Wassermann test will be considered. In this connection, the work of Dean (3) needs quoting inasmuch as in his studies on the effect of temperature on the fixation of complement, he employed a specific antigen—horse serum. This investigator employed a 1 hour fixation period at temperatures of 0°, 17°, and 37° C., and found uniformly more complement fixed at 0° C. than at 17° C. and more at 17° C. than at 37° C. This work is of especial interest in view of the fact that in accordance with the prevailing opinion (4, 5) the phenomenon of fixation of complement takes place far more readily at water bath than at colder temperatures.

EXPERIMENTAL.

The plan of these studies was, first to establish the presence of specific complement-fixing antibodies in the sera of rabbits previously immunized with purified proteins; second, to determine by means of complement fixation tests with the immune sera and specific protein antigens, the rate of fixation of complement, and to what extent this rate is affected by different temperatures.

The antigens employed were edestin, obtained from hemp-seed, and phaseolin, obtained from the kidney bean.[1] Three fixation tem-

[1] These were kindly furnished by Dr. Thomas B. Osborne of the Connecticut Agricultural Experiment Station.

peratures were adhered to: ice box (8–12°C.), room (18–23°C.), and water bath (37.5°C.). The degree of fixation was determined every 15 minutes during the 1st hour, at half hour periods during the 2nd hour, followed by determinations every hour until 5 or 6 hours had passed.

Methods of Immunization.

Three different methods of immunization were resorted to in order to vary the antibody content in the rabbit sera. Rabbit A received five intravenous injections of edestin at 48 hour intervals. The quantities of protein injected were 50, 75, 100, 125, and 150 mg. respectively. Rabbit B received five intraperitoneal injections of phaseolin at 48 hour intervals, the quantities being 100, 150, 200, 250, and 300 mg. respectively. Rabbit C received three intraperitoneal injections of phaseolin at 24 hour intervals, the quantities being 100, 150, and 200 mg. respectively.

Both edestin and phaseolin are insoluble in water but soluble in weak alkaline solutions. This necessitated the addition of a few drops of 0.1N sodium hydroxide to the proteins in order to get them in solution. This was done in each case just before the injection.

Procedure of the Complement Fixation Test.

The sheep cell system was employed. All ingredients entering into the test were used in 0.1 cc. quantities except the immune rabbit serum which was graded from 0.01 to 0.0001 cc. Two units of amboceptor, two units of complement, and from two to ten units of antigen were employed.

Sheep Cells.—Sheep were bled from the jugular vein into sterile bottles containing glass beads. After defribrination by shaking, the blood was washed four times with saline solution. The final centrifugation was carried out for 14 minutes at 1,500 revolutions per minute. 5 per cent was the strength of the standard suspension.

Hemolysin.—This was prepared in the usual manner by injecting rabbits with washed sheep cells. The unit of hemolysin was determined by titrating a series of dilutions of hemolysin serum with 0.1 cc. quantities of 1:10 pooled guinea pig complements and 0.1 cc. quantities of the standard sheep cell suspension and reading the unit

after 15 minutes incubation in the water bath (6). The serum dilution aimed at was one which contained two units of hemolysin in 0.1 cc.

Complement.—Large sized guinea pigs were bled directly from the heart. The serum was drawn after permitting the blood to remain for about 20 hours in the ice box. Pooled serum from four to five guinea pigs was used with each experiment. The unit of complement was obtained by titrating gradations of 0.1 to 0.01 cc. of both single and pooled complements with two units (0.1 cc.) of amboceptor and 0.1 cc. quantities of sheep cells. The unit in this case also was read after 15 minutes incubation in the water bath.

Protein antigens.—These were prepared by weighing 10 mg. of the protein and dissolving it in 10 cc. of 0.001N sodium hydroxide solution to which was added 0.05 cc. of 0.1N sodium hydroxide. The alkali used, represents approximately the smallest quantity necessary to get the proteins into solution. 1 cc. of this antigen solution was mixed with 9 cc. of salt solution and 0.1 cc. of this final solution, corresponding to 0.01 mg. of antigen, was used in the tests. Antigenic titrations of this solution with 0.01 cc. of specific immune serum and two units of complement showed, after primary incubation for 4 hours in the ice box, complete fixation of complement with 0.02 cc. (0.002 mg. of protein) and partial fixation with 0.01 cc. (0.001 mg. of protein). Tests for anticomplementary and hemolytic properties of these protein antigens showed that neither possessed these properties with five times the quantity (0.5 cc.) of antigen used in the tests.

Immune Serum.—The immune serum was obtained in each case by bleeding the rabbits from the marginal ear vein and separating the serum after permitting the blood to remain for several hours in the ice box. Both the edestin and phaseolin rabbits showed the presence of specific complement-fixing antibodies about 10 days after the last injection of protein. The sera were inactivated in each case for 30 minutes at 56° C. before using, and were employed in the following dilutions in the tests: 0.01, 0.007, 0.004, 0.003, 0.002, 0.001, 0.0005, 0.0003, 0.0001 cc.

Complement Fixation Tests.—The tests were carried out in the usual manner, regular Wassermann tubes being employed with the various gradations of serum, 0.1 cc. (0.01 mg.) of the protein antigen, 0.1 cc. (two units) of complement, and 0.1 cc. of saline solution. After

a given fixation period 0.1 cc. of the standard sheep cell suspension and 0.1 cc. of hemolysin (two units) were added and incubated in the water bath at 37.5° C. for about 15 minutes when the serum and antigen controls would be completely hemolyzed. All readings were made after keeping the tubes in the ice box over night. The temperatures and periods of fixation were the only variables in each test.

Effect of Temperature on the Rate of Fixation of Complement.

Contrary to the accepted views that the fixation of complement takes place far more rapidly at water bath than at a lower temperature, preliminary experiments with the rabbit sera and specific protein antigens indicated no marked difference in the degree of fixation between water bath (37.5° C.) and ice box (8–12°C.) fixation temperatures. This indeed is the reason why no attempt was made in these studies to use precise temperatures in the case of ice box and room temperature fixation. Table I gives an outline of one of these experiments with edestin immune serum. 15 minutes was the time interval chosen for fixation during the 1st hour, and multiples of 15 minutes for the succeeding hours—up to 5 or 6. It is evident from this table that the degree of fixation as measured by the increase in number of positive signs is approximately the same whether ice-box or water bath fixation is employed. There is indeed a tendency for stronger fixation in the ice box than in the water bath.

The fixation experiments at water bath and room temperatures were not extended beyond 2 hours in view of the marked deterioration of complement which takes place after prolonged exposure at these temperatures.

Text-fig. 1 shows the nature of the curve with the degree of fixation as determined by the increase in number of positive signs as the ordinate and time of fixation as the abscissa.

Table II and Text-fig. 2 represent a similar experiment with phaseolin immune serum.

Tables III and IV and Text-figs. 3 and 4 represent fixation experiments carried out at water bath, room, and ice box temperatures. The tendency for somewhat stronger fixation in the ice box is shown by

these experiments; also the fixation at room temperature approximates more closely fixation in the ice box than that in the water bath.

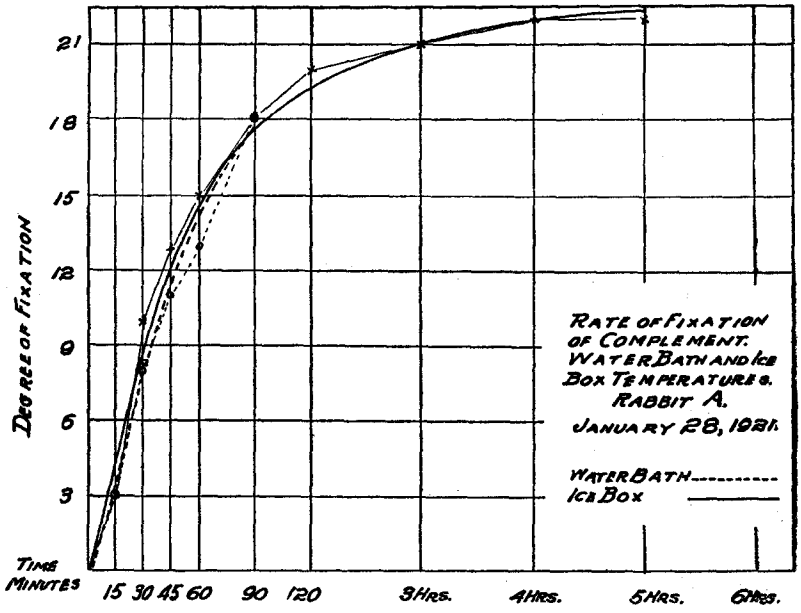
Text-figs. 5 and 6 show a marked similarity with the previous experiments and indicate to what extent the curves of fixation approximate

TABLE I.

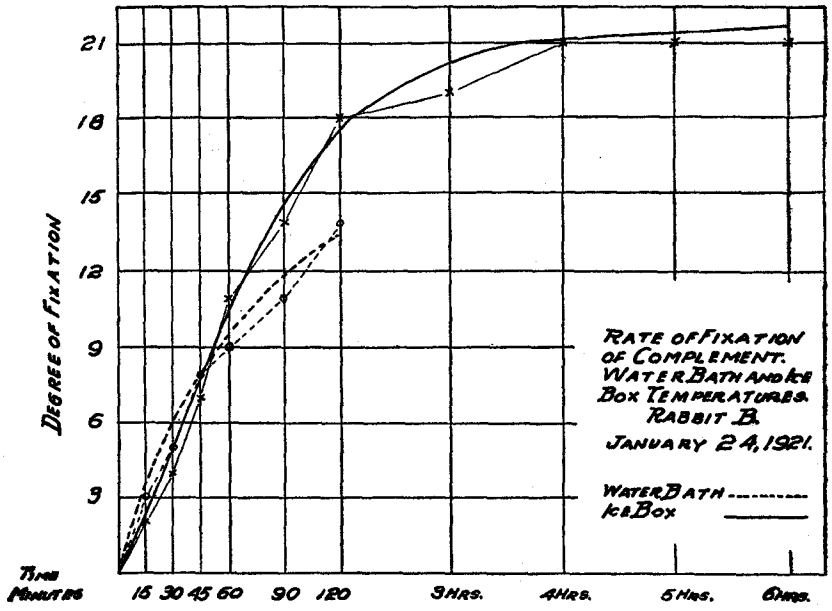
Rate of Increase of Fixation of Complement at Water Bath and Ice Box Temperatures.

Fixation.		Edestin immune serum, Rabbit A.									
Period.	Temperature.	0.01 cc.	0.007 cc.	0.004 cc.	0.003 cc.	0.002 cc.	0.001 cc.	0.0005 cc.	0.0003 cc.	0.0001 cc.	No. of positive signs denoting degree of fixation.
min.											
15	Water bath.	+	+	+	−	−	−	−	−	−	3
	Ice box.	+	+	+	−	−	−	−	−	−	3
30	Water bath.	+++	++	+	+	+	−	−	−	−	8
	Ice box.	+++	+++	++	+	+	−	−	−	−	10
45	Water bath.	++++	+++	++	+	+	−	−	−	−	11
	Ice box.	++++	++++	++	+	+	+	−	−	−	13
60	Water bath.	++++	+++	++	++	+	+	−	−	−	13
	Ice box.	++++	++++	+++	++	+	+	−	−	−	15
90	Water bath.	++++	++++	++++	+++	++	+	−	−	−	18
	Ice box.	++++	++++	++++	+++	++	+	−	−	−	18
120	Water bath.	++++	++++	++++	++++	+++	+	−	−	−	20
	Ice box.	++++	++++	++++	++++	+++	+	−	−	−	20
180	" "	++++	++++	++++	++++	+++	++	−	−	−	21
240	" "	++++	++++	++++	++++	++++	+	+	−	−	22
300	" "	++++	++++	++++	++++	++++	+	+	−	−	22

one another when the concentration of antibodies is about the same. The lesser degree of fixation at water bath temperature shown in Text-figs. 3 and 5 is difficult to explain in view of the fact that the sera of the same rabbit did not show this tendency to the same degree in Text-fig. 1.



TEXT-FIG. 1.



TEXT-FIG. 2.

Text figure 7 is given as an illustration of the nature of the curve with a weakly positive serum.

TABLE II.

Rate of Increase of Fixation of Complement at Water Bath and Ice Box Temperatures.

Fixation.		Phaseolin immune serum, Rabbit B.									
Period.	Temperature.	0.01 cc.	0.007 cc.	0.004 cc.	0.003 cc.	0.002 cc.	0.001 cc.	0.0005 cc.	0.0003 cc.	0.0001 cc.	No. of positive signs denoting degree of fixation.
min.											
15	Water bath.	++	+	−	−	−	−	−	−	−	3
	Ice box.	+	+	−	−	−	−	−	−	−	2
30	Water bath.	+++	+	+	−	−	−	−	−	−	5
	Ice box.	++	+	+	−	−	−	−	−	−	4
45	Water bath.	++++	++	+	+	−	−	−	−	−	8
	Ice box.	+++	++	+	+	−	−	−	−	−	7
60	Water bath.	++++	+++	+	+	−	−	−	−	−	9
	Ice box.	++++	+++	++	+	+	−	−	−	−	11
90	Water bath.	++++	++++	++	+	−	−	−	−	−	11
	Ice box.	++++	++++	+++	++	+	−	−	−	−	14
120	Water bath.	++++	++++	+++	++	+	−	−	−	−	14
	Ice box.	++++	++++	++++	+++	++	+	−	−	−	18
180	" "	++++	++++	++++	++++	++	+	−	−	−	19
240	" "	++++	++++	++++	++++	++++	+	−	−	−	21
300	" "	++++	++++	++++	++++	++++	+	−	−	−	21
360	" "	++++	++++	++++	++++	++++	+	−	−	−	21

A consideration of the experimental date presented in this paper indicates that the affinity for complement of specific protein antigen-antibody complexes is extremely marked, fixation of complement taking place equally as well at 8–12°C. as at 37.5°C. Theoretically this is suggestive of the close relation between the phenomenon of

precipitation and that of complement fixation, the former also taking place at cold temperatures.

TABLE III.

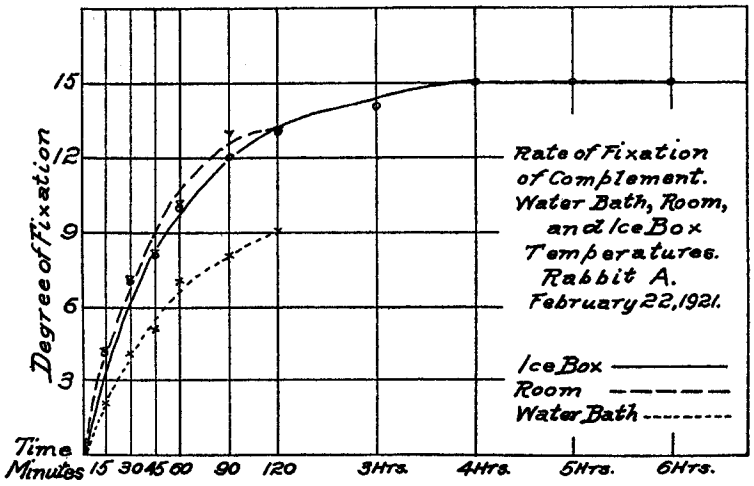
Rate of Increase of Fixation of Complement at Water Bath, Room, and Ice Box Temperatures.

Fixation.		Edestin immune serum, Rabbit A								No. of positive signs, denoting degree of fixation.
Period.	Temperature.	0.01 cc.	0.007 cc.	0.004 cc.	0.003 cc.	0.002 cc.	0.001 cc.	0.0005 cc.	0.0003 cc.	0.0001 cc.	
min.											
15	Water bath.	+	+	−	−	−	−	−	−	−	2
	Room.	++	+	+	−	−	−	−	−	−	4
	Ice box.	++	+	+	−	−	−	−	−	−	4
30	Water bath.	++	+	+	−	−	−	−	−	−	4
	Room.	+++	++	+	+	−	−	−	−	−	7
	Ice box.	+++	++	+	+	−	−	−	−	−	7
45	Water bath.	++	++	+	−	−	−	−	−	−	5
	Room.	+++	+++	+	+	−	−	−	−	−	8
	Ice box.	+++	+++	+	+	−	−	−	−	−	8
60	Water bath.	+++	++	+	+	−	−	−	−	−	7
	Room.	++++	+++	++	+	−	−	−	−	−	10
	Ice box.	++++	+++	++	+	−	−	−	−	−	10
90	Water bath.	+++	+++	+	+	−	−	−	−	−	8
	Room.	++++	++++	+++	+	+	−	−	−	−	13
	Ice box.	++++	++++	+++	+	−	−	−	−	−	12
120	Water bath.	+++	+++	++	+	−	−	−	−	−	9
	Room.	++++	++++	+++	+	+	−	−	−	−	13
	Ice box.	++++	++++	+++	+	+	−	−	−	−	13
180	" "	++++	++++	+++	++	+	−	−	−	−	14
240	" "	++++	++++	+++	++	+	+	−	−	−	15
300	" "	++++	++++	+++	++	+	+	−	−	−	15
360	" "	++++	++++	+++	++	+	+	−	−	−	15

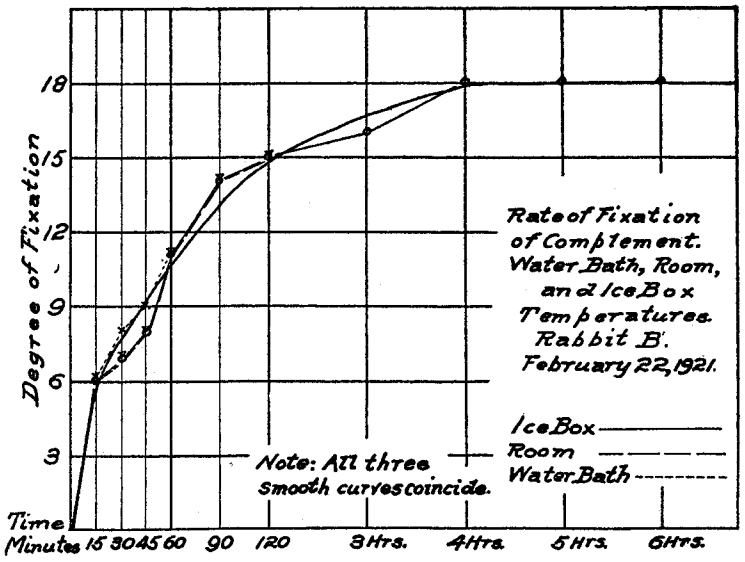
TABLE IV.

Rate of Increase of Fixation of Complement at Water Bath, Room, and Ice Box Temperatures.

Fixation.		Phaseolin immune serum, Rabbit B									No. of positive signs, denoting degree of fixation.
Period.	Temperature.	0.01 cc.	0.007 cc.	0.004 cc.	0.003 cc.	0.002 cc.	0.001 cc.	0.0005 cc.	0.0003 cc.	0.0001 cc.	
min.											
15	Water bath.	+++	++	+	−	−	−	−	−	−	6
	Room.	+++	++	+	−	−	−	−	−	−	6
	Ice box.	+++	++	+	−	−	−	−	−	−	6
30	Water bath.	+++	+++	+	+	−	−	−	−	−	8
	Room.	+++	++	+	+	−	−	−	−	−	7
	Ice box.	+++	++	+	+	−	−	−	−	−	7
45	Water bath.	++++	+++	+	+	−	−	−	−	−	9
	Room.	+++	+++	+	+	−	−	−	−	−	8
	Ice box.	+++	+++	+	+	−	−	−	−	−	8
60	Water bath.	++++	++++	++	+	−	−	−	−	−	11
	Room.	++++	++++	++	+	−	−	−	−	−	11
	Ice box.	++++	++++	++	+	−	−	−	−	−	11
90	Water bath.	++++	++++	+++	++	+	−	−	−	−	14
	Room.	++++	++++	+++	++	+	−	−	−	−	14
	Ice box.	++++	++++	+++	++	+	−	−	−	−	14
120	Water bath.	++++	++++	+++	++	+	+	−	−	−	15
	Room.	++++	++++	+++	++	+	+	−	−	−	15
	Ice box.	++++	++++	+++	++	+	+	−	−	−	15
180	" "	++++	++++	+++	+++	+	+	−	−	−	16
240	" "	++++	++++	++++	+++	++	+	−	−	−	18
300	" "	++++	++++	++++	+++	++	+	−	−	−	18
360	" "	++++	++++	++++	+++	++	+	−	−	−	18

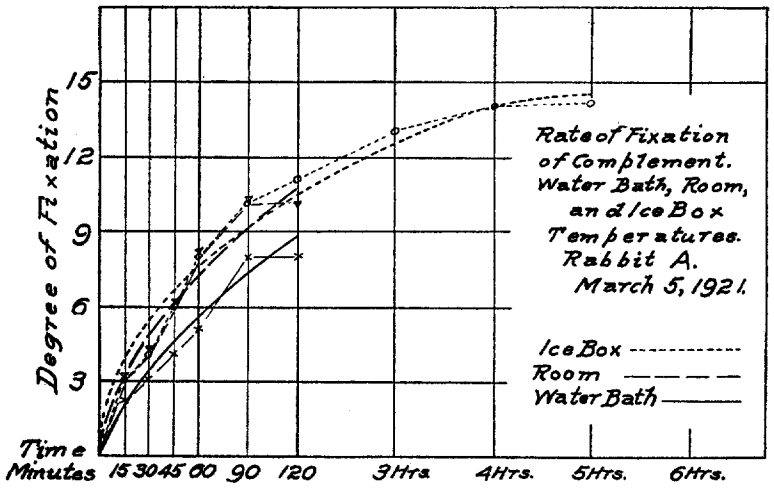


TEXT-FIG. 3.

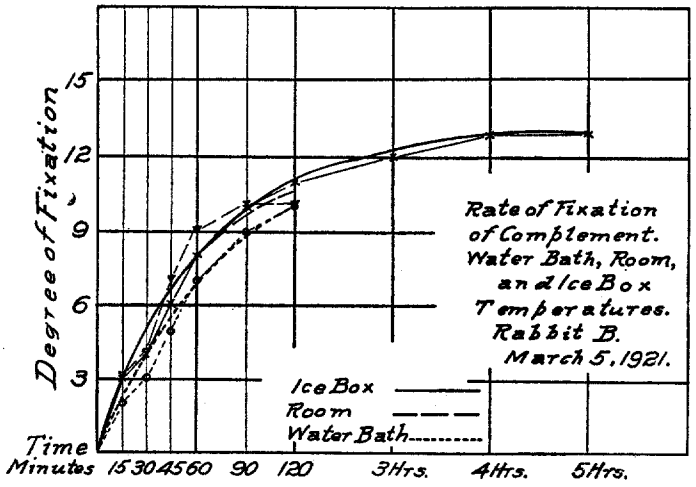


TEXT-FIG. 4.

These experiments also indicate that the rate of fixation of complement is directly proportional to the concentration of antibodies in



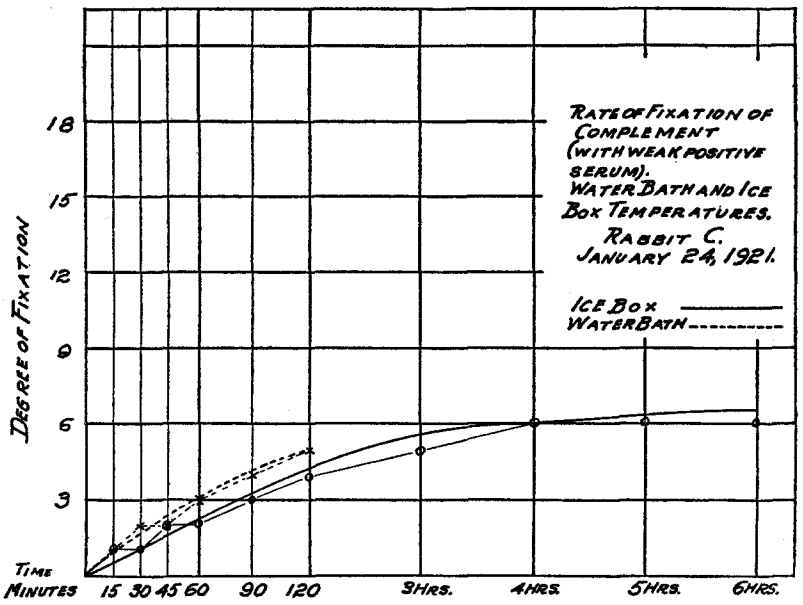
TEXT-FIG. 5.



TEXT-FIG. 6.

the immune serum. This is evident by observing the various curves; the greater the concentration of antibodies, the steeper the curve.

The rate of fixation of complement at different temperatures with various Wassermann antigens and syphilitic sera will be reported in forthcoming studies.



TEXT-FIG. 7.

CONCLUSIONS.

It is shown by complement fixation studies with protein antigens and specific immune rabbit sera that the rate of fixation of complement is determined by the concentration of antibodies in the immune sera, that the greater part of fixation of complement takes place during the 1st hour, and that fixation is practically completed at the end of 4 hours at ice box temperature.

It is further shown that the rate of fixation of complement is practically the same at ice box, room, and water bath temperatures, the tendency being for slightly stronger fixation at ice box temperature.

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