THE INFLUENCE OF TEMPERATURE ON THE FIXATION OF COMPLEMENT.[1]

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Introductory Remarks.

The experiments which are described in this paper were undertaken for the following reasons:—

It is well known that when a serum is mixed with its homologous antiserum a precipitate is formed. It is also well known that a mixture of serum and antiserum has the property of fixing or binding complement. It has been observed that under certain conditions a mixture of serum and antiserum, in which an abundant precipitate is formed, has little or no power of binding complement. On the other hand, it has been found that complement may be efficiently fixed by a mixture of serum and antiserum which appears to remain perfectly clear.

With the object of gaining information as to the relation of the fixation of complement to the formation of a precipitate the following experiments were performed (Dean, 1911 [6]):—

A series of dilutions of goat serum was prepared. To each of these dilutions of goat serum was added an equal quantity of a constant dilution of antiserum (rabbit *v.* goat). A series of mixtures was thus obtained, each of which contained the same quantity of the antiserum, while the quantity of the antigen varied. In those mixtures which contained antigen and antibody in suitable proportions precipitation was very rapid. Turbidity appeared immediately after the antibody had been mixed with the antigen. Within ten minutes the precipitate had become aggregated and formed particles visible to the naked eye. Within half an hour large flocculi had formed and had fallen to the bottom of the tube, leaving a clear supernatant fluid. In those tubes which contained a smaller quantity of antigen precipitation was delayed. Turbidity slowly appeared and slowly gave place to a condition in which the individual particles were visible. When complement was added to such a series of mixtures it was found that little or no complement was bound in those tubes in which precipitation was very rapid. When, however, the mixture contained a smaller quantity of antigen, and the precipitation process was delayed, complement was very efficiently fixed. This result was con-

[1] [Received December 22, 1916.] The majority of the experiments included in this paper were embodied in a communication to the Pathological Society at the Newcastle meeting, June 1912.

stantly obtained, provided that the antiserum was sufficiently good to produce a large flocculent precipitate when mixed with an optimal quantity of antigen.

In another experiment three exactly similar sets of antigen dilutions were prepared. To set A was added complement and then antiserum. In this set complement was present at the moment when the antigen was mixed with the antiserum. To set B was added antiserum, and half an hour was allowed to elapse before the complement was added. In the case of set C, one hour was allowed to pass between the addition of antiserum and the addition of complement. Each set was incubated for exactly one hour after the complement had been added. At the expiration of the hour allowed for fixation, blood and hæmolytic serum were added to each set, and a further period of two hours for hæmolysis followed. The result showed that complement is most effectively bound when it is present at the moment when antigen is mixed with antibody. If an interval is allowed to elapse before complement is added much less fixation takes place.

These experiments appeared to show that complement is fixed during the earliest stages of the reaction which takes place when antigen is mixed with antibody. After a visible precipitate has formed little or no complement is fixed. In mixtures which contain a relative excess of antiserum precipitation is delayed, and the earlier stages of the process of precipitation occupy sufficient time to allow the complement to be bound. Complement is in fact fixed during the early stage before a precipitate becomes visible.

It was thought that if the formation of a precipitate was delayed by cold there would be an increased fixation of complement.

Summary of Papers dealing with the Influence of Temperature on the Wassermann Reaction.

The effect of variations in temperature on the fixation of complement does not appear to have attracted much attention. Few papers have been published and these have been devoted to the Wassermann reaction.

Satta and Donati (1909 [17]) were the first to publish observations on this subject. The fixation of complement in the Wassermann reaction was investigated at various temperatures. I have not been able to obtain their paper and the abstract is not very full. Jacobsthal (1910 [13]) pointed out that, in carrying out the Wassermann test, better fixation was obtained at 4° C. than at 37° C. In a series of 200 sera the reactions obtained by fixation at 4° C. and 37° C. were compared. Jacobsthal obtained 2 per cent. more positive results by fixation in the cold. Guggenheimer (1911 [11]) used 623 sera and compared the results obtained by fixation at 0° C. and 37° C. Two hundred and fifty-five gave a positive reaction by both methods and of these thirty-two gave a stronger reaction after incubation at 37° C. and thirty-seven after incubation at 0° C. In the remaining 186 no difference could be observed in the results obtained by the two methods. Three hundred and forty-eight gave a negative result by both methods. Eight sera gave a positive by the warm method and a negative reaction by the cold method. Twelve sera gave a negative by the warm method and a positive reaction by the cold method. The total number of positive reactions, after fixation at 37° C., was 263, after fixation at 0° C. was 267. Guggenheimer also stated that the extract which he used fixed more complement at 0° C. than at 37° C. Guggenheimer did not, however, consider that the results obtained could be explained

by the increased anti-complementary action of the extract at the lower temperature, and in his series of experiments precautions were taken to avoid any error due to this factor.

Altmann and Zimmern (1912 [2]) have described the results obtained by the examination of 1902 sera. Of these 1610 reacted alike by both methods. Of the remaining 292, 121 gave absolutely different results by the two methods. Seventy-six gave positive results by the cold and negative by the warm method. Forty-five were negative by the cold and positive by the warm method. The remaining 171 showed only qualitative differences. Ninety-six gave stronger reactions after fixation in the cold, seventy-five after fixation at the warmer temperature. In a second paper (1913 [1]) Altmann has paid particular attention to the stage of the disease at which the specimen was obtained. He found that fixation by the usual method, at 37° C., gave a higher percentage of positive results during the primary and early secondary periods. On the other hand, during the tertiary and latent periods, more positive results were obtained if the first part of the reaction was carried out at 4° C.

EXPERIMENTAL OBSERVATIONS.

EXPERIMENT 1.—Table I. (Part 1).

PRECIPITATION EXPERIMENT.

1 C.c. of Normal Horse Serum (Antigen), Diluted.	+ 1 C.c. of Rabbit *v.* Horse Serum (Antiserum), Diluted 1 in 5.		
	A. 0° C.	B. 17° C.	C. 37° C.
(1) 1 in 5 .	Slightly turbid.	Uniform turbidity.	Few flocculi.
(2) 1 ,, 10 .	Turbid.	Small particles.	Small deposit.
(3) 1 ,, 20 .	Very turbid.	Large flocculi.	Large deposit.
(4) 1 ,, 40 .	,, ,,	,, ,,	,, ,,
(5) 1 ,, 80 .	,, ,,	Deposit and clear fluid.	,, ,,
(6) 1 ,, 160 .	,, ,,	,, ,, ,,	Deposit.
(7) 1 ,, 320 .	Turbid.	Large flocculi.	Small deposit.
(8) 1 ,, 640 .	Slightly turbid.	Small particles.	Large flocculi.
(9) 1 ,, 1280 .	Opalescent.	Turbid.	Turbid.
(10) 1 ,, 2560 .	Trace opalescence.	Opalescent.	Opalescence.
(11) 1 ,, 5120 .	?Trace opalescence.	Trace opalescence.	,,

This Table shows the amount of precipitation after one hour.

Three sets of horse-serum dilutions were prepared. A sufficient quantity of the antiserum dilution was prepared and divided into three portions. Set A of the horse-serum dilutions, together with a tube of diluted antiserum, was placed in the cold at a temperature of 0° C. Set C, together with a tube of diluted antiserum, was placed in the incubator at a temperature of 37° C. Set B remained in the laboratory at a temperature of 17° C. One hour was allowed for the ingredients to acquire the required temperature. The diluted antiserum was then added to the various antigen dilutions. Set A was mixed at 0° C., set B at 17° C., and set C at 37° C.

In set A clouding was noticed almost immediately in tubes 3, 4, 5, and 6, and within a few minutes in tubes 2 and 7. The state of affairs after one hour at 0° C. is recorded in the table. Tubes 1 to 10 showed various grades of turbidity. The turbidity in these tubes was quite uniform and no particle formation could be detected. In set B turbidity appeared almost immediately in tubes 3, 4, 5, and 6. After fifteen minutes small particles were visible in

tubes 4, 5, and 6; and tubes 2, 3, 7, 8, and 9 showed various grades of uniform turbidity. The appearances, after one hour at 17° C., are recorded in the table.

In set C turbidity appeared almost immediately in tubes 2 to 8, and after ten minutes tubes 3, 4, and 5 showed a definite deposit with a clear supernatant fluid. The appearances, after one hour at 37° C., are shown in Table I., Part 1.

EXPERIMENT 1.—Table I. (Part 2).

COMPLEMENT FIXATION EXPERIMENT.

0·5 C.c. of Horse Serum (Antigen), Diluted.	+ 0·5 C.c. Rabbit v. Horse (Antiserum), Diluted 1 in 5.								
	+ 0·5 C.c. Complement, Diluted 2 in 5.			+ 0·5 C.c. Complement, Diluted 1 in 5.			+ 0·5 C.c. Complement, Diluted 1 in 10.		
	0° C.	17° C.	37° C.	0° C.	17° C.	37° C.	0° C.	17° C.	37° C.
(1) 1 in 5	■	■	■	■	■	■	■	◪	◪
(2) 1 ,, 10	◪	◪	◪	◪	◪	◪	◪	◪	◪
(3) 1 ,, 20	◧	◧	◧	◪	◪	◪	◪	◪	◪
(4) 1 ,, 40	◪	◪	◪	◪	◪	◪	◪	◪	◪
(5) 1 ,, 80	◪	◪	◪	◪	◪	◪	□	◪	◪
(6) 1 ,, 160	◪	◪	◪	◪	□	□	□	□	□
(7) 1 ,, 320	◪	◪	◪	□	□	◪	□	□	□
(8) 1 ,, 640	◪	◧	■	□	□	■	□	□	◪
(9) 1 ,, 1280	◪	■	■	◪	◪	■	□	□	◪
(10) 1 ,, 2560	■	■	■	◧	■	■	◪	◪	■
(11) 1 ,, 5120	■	■	■	■	■	■	■	■	■

SCHEME TO ILLUSTRATE THE DIAGRAMS USED IN THE TABLES.

No hæmolysis	□
Trace	◪
Slight	◪
Half hæmolysed	◧
Marked hæmolysis	■
Almost complete hæmolysis .	■
Complete	■

The horse serum (antigen) and the antiserum used in this experiment were exactly the same as those used in the experiment shown in Table I., Part 1. The dilutions were the same, and the experiment was set up under the same conditions and as soon after the precipitation experiment as possible.

Nine rows of antigen dilutions were prepared and divided into three sets. There were also prepared three dilutions of fresh guinea-pig serum (complement), 2 in 5, 1 in 5, and 1 in 10, and a sufficiency of the antiserum in a dilution of 1 in 5. The diluted antiserum and the three dilutions of complement were divided into three lots. Set A of antigen dilutions was placed at a temperature of 0° C., set B was allowed to stand at room temperature, 17° C., and set C was placed at a temperature of 37° C. With each set of antigen dilutions was placed a set of complement dilutions and a tube of diluted antiserum. A period of one hour was allowed for all the ingredients to come to the required temperature. Complement and antiserum were then added to each tube. The tubes were then allowed one hour at the three temperatures (0° C., 17° C., and 37° C.), for complement fixation to take place. A mixture of an emulsion of sheep corpuscles, with an equal quantity of diluted antiserum (rabbit *v.* sheep), was then added to each tube. The hæmolytic serum had been titrated immediately before the experiment, and the quantity added was equivalent to three minimal hæmolytic doses for this quantity of emulsion of corpuscles, acting in conjunction with the 1 in 10 dilution of guinea-pig serum. The three sets of tubes were then placed in the incubator at a temperature of 37° C. After set B and set C had been left in the incubator for two hours and set A for two and a half hours, the results (Table I., Part 2) were read and recorded. The extra half-hour was allowed to set A, in the endeavour to compensate for the delay in hæmolysis which was to be expected, while the temperature in the tubes of this set rose from 0° C. to 37° C. As a matter of fact, no change occurred in any tube of the three sets after the expiration of the first hour and a half.

Remarks on Experiment 1.—The first part of the experiment showed that the rate of precipitation was very distinctly retarded by cold and accelerated by heat. Moreover, at the end of the hour, the difference in the size of the particles in the three sets was very evident. In set A the process had in no tube proceeded to the point at which separate particles were visible to the eye. In sets B and C distinct particles or large flocculi had formed in the majority of the tubes and in many the aggregations of precipitate had reached such size that they had fallen, to form a deposit at the bottom of the tube. The influence of the relative proportions of antigen and antibody in the mixture was well seen in these three sets of tubes. In tubes 3, 4, and 5, the quantities of antigen were favourable to rapid precipitation, and the process in these tubes was complete in set C, and almost complete in set B, at the end of one hour. These tubes formed a zone in which the proportions were optimal for precipitation. In tubes 7–11 precipitation was delayed by deficiency of antigen. In tubes 1 and 2 interference and delay were produced by a relative excess of antigen. On turning to the second part of the table it will be seen that the best fixation of complement occurred in tubes 5, 6, and 7, that is to say, in tubes which contained somewhat less antigen than the quantity most favourable to precipitation. Tubes 7, 8, 9, and 10 show in a marked fashion the increased fixation of complement effected at the

lower temperature. More fixation has taken place at 0° C. than at 17° C., and more at 17° C. than at 37° C. In tubes 3, 4, 5, and 6 there is no evident difference in the results obtained at the three temperatures. On reference to the description of the first part of the experiment it will be seen that turbidity was observed immediately after mixture in these tubes, even at 0° C. The results observed in these tubes confirm the view that the period of optimal fixation is over when the aggregation of particles has proceeded so far as to produce visible turbidity. It is in the very earliest stages of precipitation, when the precipitate is in a very fine state of division and presents a very large surface, that the conditions are most favourable to complement fixation. Tubes 7, 8, 9, and 10 contained less antigen, with the result that the precipitation was relatively slow. In these tubes the effect of cold was to prolong very effectively the early stages of precipitation, with the result that complement was very effectively fixed. It is worthy of note that in the case of tubes 1 and 2 no difference in the amount of complement fixed in the three sets was observed.

EXPERIMENT 2.

Ten sets of dilutions of an antigen (horse serum) from 1 in 10 to 1 in 10,240 were prepared. Five of these sets were cooled to 0° C. and five were warmed to 37° C. Five dilutions of the homologous antiserum were prepared, namely, 1 in 5, 1 in 10, 1 in 20, 1 in 40, 1 in 80, and 1 in 160. One-half of each of these dilutions was warmed to 37° and one-half cooled to 0° C. A 1 in 10 dilution of fresh guinea-pig serum was prepared. One-half of this was warmed to 37° C., and one-half cooled to 0° C. The various dilutions of antigen and antiserum and the diluted complement were thus brought to the two required temperatures, 37° C. and 0° C. Antigen, antibody, and complement were then mixed in the manner shown in Table II. (p. 199). Five sets prepared from previously warmed ingredients were left at a temperature of 37° C. for half an hour. The other five sets, prepared from cold ingredients, were left at 0° C. for half an hour. At the end of this time both sets were placed on a bench in the laboratory and allowed to remain at room temperature (16° C.), for a further period of one hour. An emulsion of sheep corpuscles was then mixed with an equal amount of a dilution of the homologous hæmolytic serum. This dilution contained three minimal hæmolytic doses. Of this mixture 1 c.c. was added to every tube. All the tubes then contained 2·5 c.c., of which 0·5 c.c. was diluted antigen, 0·5 c.c. diluted antiserum, and 0·5 c.c. diluted complement. All the tubes were then placed in an incubator at 37° C. and allowed to remain at this temperature for two hours. The results are shown in Table II.

[TABLE

EXPERIMENT 2.—Table II.

0·5 C.c. of Horse Serum (Antigen), Diluted.	+ 0·5 C.c. Antiserum, 1 in 10.		+ 0·5 C.c. Antiserum, 1 in 20.		+ 0·5 C.c. Antiserum, 1 in 40.		+ 0·5 C.c. Antiserum, 1 in 80.		+ 0·5 C.c. Antiserum, 1 in 160.	
	37° C.	0° C.	37° C.	0° C.	37° C.	0° C.	37° C.	0° C.	37° C.	0° C.
(1) 1 in 10	■	■	■	■	■	■	■	■	■	■
(2) 1 ,, 20	■	■	■	■	■	■	■	■	■	■
(3) 1 ,, 40	■	■	■	■	■	■	■	■	■	■
(4) 1 ,, 80	□	□	■	■	■	■	■	■	■	■
(5) 1 ,, 160	□	□	■	■	■	■	■	■	■	■
(6) 1 ,, 320	□	□	■	□	■	□	■	■	■	■
(7) 1 ,, 640	■	□	■	□	■	□	■	■	■	■
(8) 1 ,, 1280	■	■	■	□	■	□	■	□	■	■
(9) 1 ,, 2560	■	■	■	□	■	□	■	■	■	■
(10) 1 ,, 5120	■	■	■	■	■	■	■	■	■	■
(11) 1 ,, 10,240	■	■	■	■	■	■	■	■	■	■

Far more fixation of complement occurred in the sets which were mixed at 0° C. than in the sets mixed at 37° C. With each of the five antiserum dilutions the better result was obtained by mixing in the cold. The difference is best marked in those tubes which contain relatively small amounts of antigen. The results suggested that retardation of the rate of precipitation by cold might not be the essential cause of the increased fixation of complement at the lower temperature. The results are consistent with the view that fixation of complement is the result of the formation of an adsorption compound. The formation of this adsorption compound is favoured by the lower temperature. The table also shows the influence of relative proportions of antigen and antibody on complement fixation. With an antiserum dilution of 1 in 20 the best fixation was obtained with antigen diluted 1 in 160, antiserum diluted 1 in 40 required a 1 in 320 dilution of antigen, antiserum 1 in 80 required antigen 1 in 640, and antiserum 1 in 160 required antigen 1 in 1280.

EXPERIMENT 3.

This experiment shows that similar results may be obtained with a bacterial antigen and its homologous antiserum.

The method employed was essentially the same as that employed in the last experiment, except that three dilutions of complement and only one dilution of antiserum were employed. Duplicate sets of all ingredients were prepared. Set A was warmed to 37° and set B was cooled to 0° C. Antigen, antibody, and complement were then mixed in the proportions shown in Table III. (p. 201). Set A was then kept for half an hour at 37° C. and set B for half an hour at 0° C. Both sets were then exposed to room temperature for one hour. Blood and hæmolytic serum were added and all the tubes were incubated at 37° C. for two hours.

The results showed a very marked degree of difference between the two sets. The favourable influence of the lower temperature of fixation was best seen in the tubes which contained the 1 in 5 dilution of guinea-pig serum.

EXPERIMENT 4.

The experiments already described show that in mixtures of antigen, antibody, and fresh guinea-pig serum there is a more effective fixation of complement at 0° C. than at 37° C. Now precipitation is delayed at 0° C. and a similar retardation of complement fixation might be expected at the lower temperature. In the above-described experiments the period allowed for fixation was the same for both temperatures. It seemed probable that one hour was too short a time to allow for the maximum fixation to be effected. If it could be shown that the maximum complement

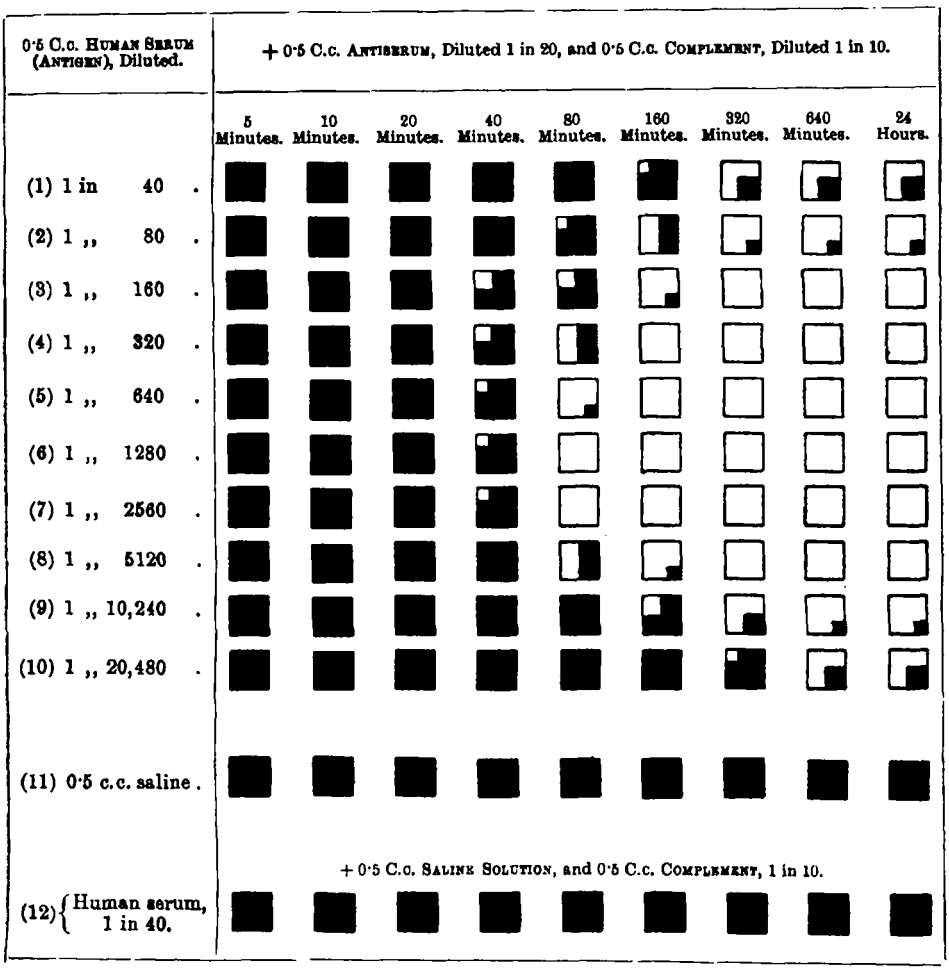
EXPERIMENT 3.—Table III.

0·5 C.c. Extract of B. Typhosus, Diluted.	+ 0·5 C.c. Antityphoid Serum, Diluted 1 in 100.						+ 0·5 C.c. Saline.	
	Complement 2 in 5.		Complement 1 in 5.		Complement 1 in 10.		Complement 1 in 10.	
	0° C.	37° C.	0° C.	37° C.	0° C.	37° C.	0° C.	37° C.
(1) 1 in 5								
(2) 1 ,, 10								
(3) 1 ,, 20								
(4) 1 ,, 40								
(5) 1 ,, 80								
(6) 1 ,, 160								
(7) 1 ,, 320								
(8) 0·5 c.c. saline								

fixation was not attained within one hour at 0° C., it was obvious that the advantage of the lower temperature was still greater than that shown in the first three tables.

In this experiment ten dilutions of the antigen (normal human serum) were employed. Nine sets of antigen dilutions were exposed to a temperature of 0° C., together with tubes containing a sufficiency of the homologous anti-

EXPERIMENT 4.—Table IV.



serum (in a dilution of 1 in 20), and of fresh guinea-pig serum (in a dilution of 1 in 10). Three-quarters of an hour were allowed for the ingredients to cool down. Then to each tube was added 0·5 c.c. of diluted antiserum and 0·5 c.c. of diluted complement. After the ingredients had been mixed, each set was allowed to remain at 0° C. for the number of minutes shown in the table. At the expiration of such period the particular set was removed to the laboratory, and blood and hæmolytic serum were added to each tube. The set was then incubated for one hour at 37° C., and the result recorded. Table IV.

shows the results obtained after one hour had in each case been allowed for hæmolysis, at the end of which period all the tubes of the first three sets showed complete hæmolysis. In all three sets, however, tubes 3, 4, 5, 6, and 7 showed delay in the onset of hæmolysis, which no doubt indicated that a certain degree of complement fixation was effected during the shorter periods of time.

In this experiment the quantities of antibody and complement were kept constant in each tube. Various quantities of antigen were employed and various periods of time, from five minutes to twenty-four hours, were allowed for fixation. The experiment thus afforded the opportunity of observing the rate of the reaction in a series of mixtures containing antigen and antibody in various proportions. The results showed that the rate of the reaction depended on the proportion of antigen to antibody present in the mixture. Tubes 1 to 3 contained a relative excess of antigen and tubes 8 to 10 a relative excess of antibody. In these tubes complement fixation was relatively slow. This result depends, no doubt, on the slowing of precipitation which results from relative excess of either antigen or antibody. In the presence of excess of either ingredient the precipitate is less in amount and is formed slowly. Under these conditions less complement is fixed and the process of fixation requires a relatively long time. In tube 2 the maximum was reached in 320 minutes, in tubes 1, 9, and 10 in 640 minutes. In the remaining tubes the time required for maximum fixation could not be determined as the dose of complement employed was too small for the purpose. The tubes numbered 11 and 12 were controls and showed that neither antigen nor antibody fixed any appreciable quantity of complement during twenty-four hours at 0° C.

EXPERIMENTS 5 and 6.

These experiments were undertaken to compare the amount of complement fixed and the rate of the reaction at 0° C. and at 37° C.

To obtain a quantitative measure five doses of complement were employed. Each tube of the experiment proper contained 0·5 c.c. of human serum (the antigen), in a dilution of 1 in 640 and 0·5 c.c. of antiserum in a dilution of 1 in 20.

The experiments necessitated the use of a very elaborate series of control tubes. These controls, which form a very essential part of the experiments, are shown in the tables. It is sufficient to observe that no alteration in the complement was observed, and no complement was fixed by either antigen or antibody, acting alone, during the time limits of the experiments. It was not possible to carry out both experiments on the same day.

The experiment shown in Table VI. was done on the day following that shown in Table V. Exactly the same antigen and antibody, hæmolytic serum, and sheep corpuscles were employed. A different guinea-pig serum was used for complement on the second day. Every effort was made to make the two experiments comparable.

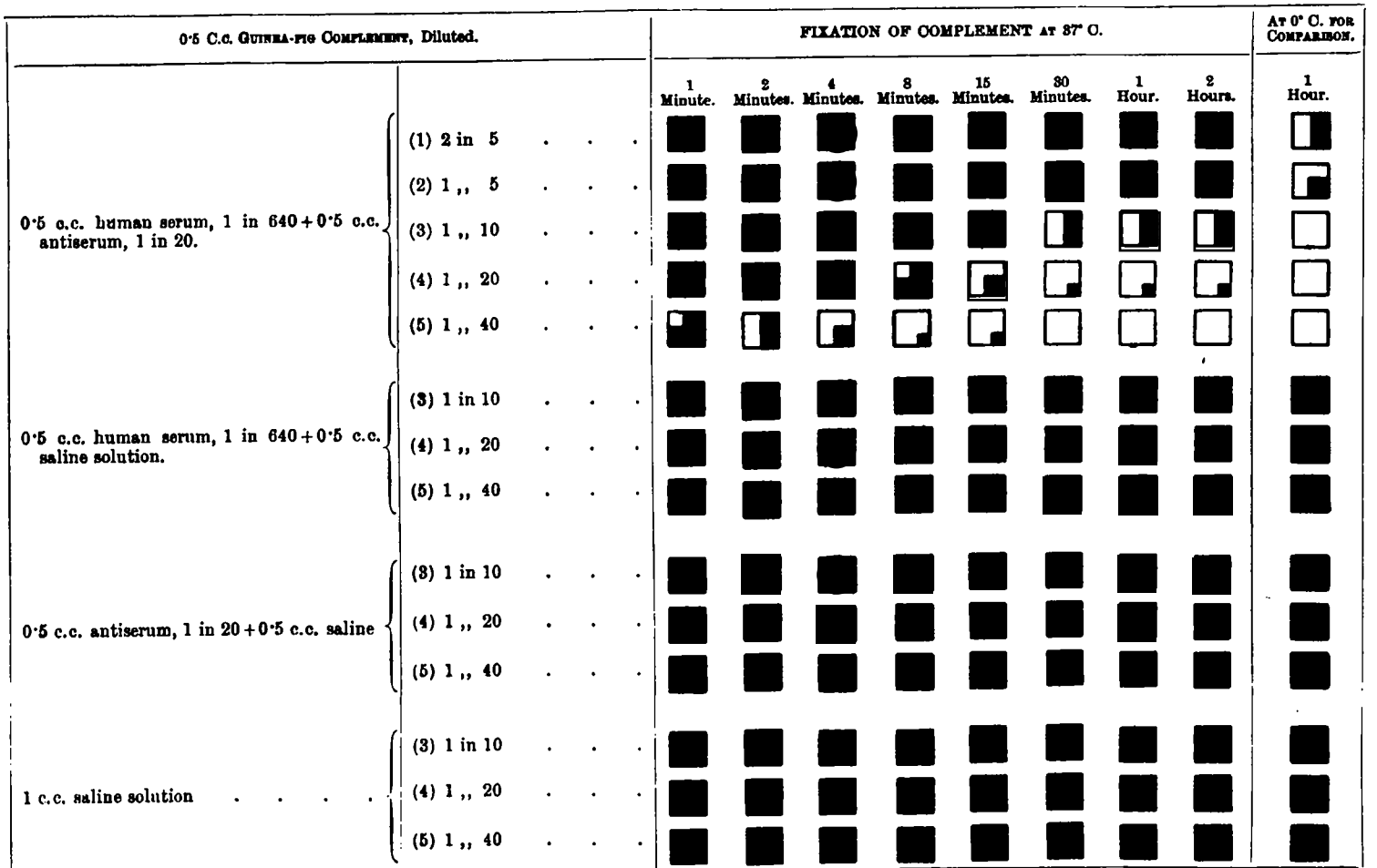
In the second experiment (Table VI.), one set was allowed to fix at 0° C. for

one hour for purposes of comparison. It will be seen that the result obtained in this set agrees fairly closely with that of the similar set (fixation for one hour) in Table V.

EXPERIMENT 5.—Table V.

+ 0·5 C.c. Guinea-pig Serum Complement, Diluted.		FIXATION, 0° C.c.					
		7½ Minutes.	15 Minutes.	30 Minutes.	1 Hour.	2 Hours.	4 Hours.
0·5 c.c. human serum, diluted 1 in 640 + 0·5 c.c. antiserum, diluted 1 in 20.	(1) 2 in 5 .				■	■	■
	(2) 1 ,, 5 .	■	■	■	▣	□	□
	(3) 1 ,, 10 .	▣	▣	▣	□	□	□
	(4) 1 ,, 20 .	▣	▣	□	□	□	□
	(5) 1 ,, 40 .	▣	□	□	□	□	□
0·5 c.c. human serum, diluted 1 in 640 + 0·5 c.c. saline.	(1) 2 in 5 .				■	■	■
	(2) 1 ,, 5 .	■	■	■	■	■	■
	(3) 1 ,, 10 .	■	■	■	■	■	■
	(4) 1 ,, 20 .	■	■	■	■	■	■
	(5) 1 ,, 40 .	■	■	■	■	■	■
0·5 c.c. antiserum, diluted 1 in 20 + 0·55 c.c. saline.	(1) 2 in 5 .				■	■	■
	(2) 1 ,, 5 .	■	■	■	■	■	■
	(3) 1 ,, 10 .	■	■	■	■	■	■
	(4) 1 ,, 20 .	■	■	■	■	■	■
	(5) 1 ,, 40 .	■	■	■	■	■	■
1 c.c. saline.	(1) 2 in 5 .				■	■	■
	(2) 1 ,, 5 .	■	■	■	■	■	■
	(3) 1 ,, 10 .	■	■	■	■	■	■
	(4) 1 ,, 20 .	■	■	■	■	■	■
	(5) 1 ,, 40 .	■	■	■	■	■	■

EXPERIMENT 6.—Table VI.



In the case of the experiment recorded in Table V., the sets of complement dilutions and flasks containing the diluted antiserum and the diluted antibody were cooled for one hour at 0° C. The antigen and antibody were then added to the tubes containing the complement. At the expiration of the time shown in the table each set was removed, a mixture of corpuscles and diluted hæmolytic serum (containing three minimal hæmolytic doses) was added and a period of two hours was allowed for hæmolysis at 37° C.

The experiment shown in Table VI. was carried out in similar fashion. All ingredients were allowed to stand for half an hour at 37° C. Antigen, antibody, and complement were then mixed in the proportions shown in the table.

After the time indicated in the table a mixture of blood and hæmolytic serum (containing three minimal doses) was added to each set and an hour was allowed for hæmolysis. For purposes of comparison one set was allowed one hour for fixation at 0° C. and was treated exactly as the sets of the experiment of the previous day (Table V.).

It is evident from these two experiments that very much more complement is fixed at 0° C. than at 37° C. The maximum fixation was attained more slowly at 0° C. than at 37° C. The reaction was complete at 37° C. in half an hour, while at 0° C. considerable progress was made after the expiration of the second hour. If the results at the two temperatures at the end of one hour (the usual period for fixation) are compared, the advantage is on the side of the set allowed to fix at the low temperature. If the full time allowed for the maximum fixation at 0° C. is allowed, the difference is still more marked. If suitable proportions of antigen and antibody are employed, fixation at 0° C. is considerable even if only a short interval is allowed after mixing. The seven and a half minutes set in Table V. compares favourably with the eight minutes set in Table VI. The fact that the reaction is slower but more complete at 0° C. than at 37° C. is in favour of the view that the process depends on the formation of an adsorption compound. A chemical union between complement and amboceptor, such as is implied by the side-chain hypothesis, should be more complete at the higher temperature.

EXPERIMENT 7.

Table VII. (p. 207) represents the results of a Wassermann reaction. The ingredients were a known positive serum and an alcoholic extract of liver from a case of congenital syphilis.

Five dilutions of serum and three dilutions of extract were employed. Each quantity of serum was mixed with each quantity of extract so that the mixtures containing various proportions of extract and serum were obtained. The general method followed was similar to that employed in previously described experiments. The ingredients for one set (A) were cooled to 0° C., mixed, and allowed to stand for one hour at 0° C. The tubes were then removed to the incubator and allowed a second hour for fixation at 37° C. The ingredients for the other set (B) were allowed to stand for one hour at room temperature (18° C.). They were then mixed and allowed one hour at

room temperature for fixation. The tubes were then removed to the incubator and allowed a second hour at 37° C. At the expiration of the second hour after the ingredients had been mixed, blood corpuscles and hæmolytic serum were added and two hours were allowed for hæmolysis. The complement employed in this experiment was a 1 in 10 dilution of fresh guinea-pig serum. Each tube received 0·5 c.c. of this dilution.

In the previous experiments of this series all the ingredients used were either cooled to 0° or warmed to 37° C., before they were mixed. This is necessary if it is desired to demonstrate the marked difference between the amount of complement fixed at these two temperatures. For, provided that antigen and antibody are mixed in approximately suitable proportions, the conditions most favourable to complement fixation are present immediately

EXPERIMENT 7.—Table VII.

WASSERMANN REACTION.

Syphilitic Serum, Diluted.	Dilutions of Alcoholic Liver Extract.						+0·5 C.c. Saline Serum Controls.	
	1 in 5		1 in 10		1 in 20			
	Mixed at 0° C.	Mixed at 18° C.	Mixed at 0° C.	Mixed at 18° C.	Mixed at 0° C.	Mixed at 18° C.	Mixed at 0° C.	Mixed at 18° C.
(1) 1 in 5 .								
(2) 1 „ 10 .								
(3) 1 „ 20 .								
(4) 1 „ 40 .								
(5) 1 „ 80 .								
(6) 1 „ 160 .								
(7) Extract control.								
(8) Double quantity of extract.								

after antigen and antibody have been mixed together. The usual method is to mix all the ingredients at room temperatures and then to place the tubes in an incubator at 37° C. for one hour. If the quantity in each tube is 1·5 c.c., and ordinary thick glass test tubes and wooden test tube racks are used, twenty minutes to half an hour elapses before the contents become warm. By that time the reaction is in the majority of tubes already complete. That is to say, the putting of the tubes in an incubator for an hour has very little influence on the amount of complement fixed. The intention was to compare the results obtained by fixation at 0° C. with those obtained by the ordinary methods. By the ordinary methods is implied the mixing of the ingredients at room temperature and then placing the mixtures in the incubator at 37° C.

The results shown in Table VII. afford in fact a comparison of the results of fixation at 0° C. and 18° C. As already mentioned both

sets were put in the incubator for a second hour. This was done to make sure that both sets were at the same temperature when blood and hæmolysin were added. Had there been any difference in temperature between the two sets, at the moment when blood and hæmolysin were added, hæmolysis would have been retarded in the cooler set of tubes. The results show that fixation was more marked in the set mixed and allowed to stand at 0° C. With the 1 in 5 extract dilution there was a great difference between the two tubes containing the 1 in 80 serum dilution. Similar differences with the extract dilutions 1 in 10 and 1 in 20 were observed with the serum dilutions 1 in 40 and 1 in 20 respectively. The controls showed that complement was not appreciably affected at either temperature by either the extract or the serum in any dilution employed in the experiment.

EXPERIMENT 8.—Table VIII.

WASSERMANN REACTION.

Syphilitic Serum "L," Diluted.	+ Liver Extract, Diluted 1 in 10.						Saline Solution. (Serum Control).		
	Complement Diluted 1 in 5.			Complement Diluted 1 in 10.					
	0° C.	14° C.	37° C.	0° C.	14° C.	37° C.	0° C.	14° C.	37° C.
(1) 1 in 5 . .									
(2) 1 ,, 10 . .									
(3) 1 ,, 20 . .									
(4) 1 ,, 40 . .									
Syphilitic Serum "O," Diluted.									
(5) 1 in 5 . .									
(6) 1 ,, 10 . .									
(7) 1 ,, 20 . .									
(8) 1 ,, 40 . .									
(Extract Controls.)									
(9) Single, 1 in 10									
(10) Double, 1 ,, 5									

This table records an experiment with two syphilitic sera in dilutions from 1 in 5 to 1 in 40.

Two complement doses were employed, that is to say, 1 in 5 and 1 in 10 dilutions of fresh guinea-pig serum. On this occasion only one extract dilution (1 in 10) was used. The experiment was conducted on similar lines to those already described. The necessary ingredients were divided into three sets and exposed to temperatures of 0° C., 14° C. (room temperature), and 37° C. After one hour the ingredients were mixed. Set A was left for half an hour at 0° C. and set B for half an hour at 14° C. Sets A and B were then put in the incubator at 37° C. for half an hour. Set C was allowed one hour at 37° C. At the expiration of the hour allowed for fixation blood and hæmolysin were added to the tubes of all three sets, and two hours at 37° C. were allowed for hæmolysis.

The result showed that more complement was fixed at 0° C. than at 14° C. and more at 14° C. than at 37° C.

EXPERIMENT 9.

The experiments reproduced in the preceding tables show plainly that fixation of complement is more complete at 0° C. than at 37° C. The ingredients employed are typical of those usually employed in complement fixation experiments, namely, a normal serum and its homologous antiserum, a bacterial extract and antiserum, and alcoholic organ extract and syphilitic serum. The experiments were designed to support the hypothesis that the physical state of the precipitate is the important factor in determining the amount of complement fixed. The experiment recorded in Table I. shows that when a serum is mixed with the homologous antiserum in the cold the precipitate is more slowly formed, and at the end of a given time is in a finer state of division than the precipitate formed in a similar mixture at a temperature of 37° C. The smaller particles of the cold mixture afford a larger surface for adsorption and the amount of complement fixed is in consequence greater. The smaller size of the individual particle of the precipitate is, however, not the only factor in the increased complement fixation observed at the lower temperature.

In 1905, Welsh and Chapman demonstrated that, under the usual experimental conditions, the precipitate formed in a mixture of serum and homologous antiserum was almost entirely derived from the proteins of the antiserum. This important observation which was supported by subsequent papers—Welsh and Chapman (1907 [19] and 1908 [20]), and Chapman (1910 [5])—throws considerable light, not merely on the precipitation reaction, but also on the mechanism of agglutination and complement fixation. In carrying out a precipitation test it is possible to dilute the antigen to an enormous extent, frequently to 1 in 50,000 or 100,000, in exceptional cases even to 1 in 1,000,000. It is, however, customary to use a relatively large amount of the antiserum which is commonly employed in a dilution of 1 in 2 to 1 in 10. If a smaller quantity of antiserum be chosen, for instance, a 1 in 100 dilution, little or no precipitate will be produced. The

reason for this is apparent from the experiments of Welsh and Chapman, for in such cases there is not sufficient antiserum present to produce an appreciable precipitate. In complement fixation experiments, on the other hand, relatively small quantities of the antiserum can be employed. A good antiserum can be always used in a dilution of 1 in 100, often in a dilution of 1 in 500, or 1 in 1000. If, however, a cubic centimetre of a 1 in 500 dilution of antiserum be mixed with an equal quantity of a suitable dilution of the homologous antigen, not even a trace of precipitate can as a rule be detected. Such a mixture contains far too little of the precipitable substance of the antiserum for the formation of a visible precipitate. Nevertheless, such a mixture may serve admirably for complement fixation.

Such an experiment can easily be performed with any good antiserum, and the result at first sight appears to afford evidence for the view that precipitation and complement fixation are separate and independent phenomena.

The methods usually employed in carrying out a precipitation experiment are, however, by no means suitable if it is desired to demonstrate the presence of a scanty precipitate. A few cubic centimetres of a mixture of a greatly diluted antigen with a greatly diluted antibody in an ordinary test tube may appear perfectly clear. But if a similar mixture is poured into a polarimeter tube, a very definite opalescence or even turbidity may be observed.

By this quite simple expedient precipitates may be demonstrated which could not be detected in a similar quantity of the mixture contained in an ordinary test tube. Nevertheless, the fact that complement fixation occurs in mixtures containing so small a quantity of antiserum that no precipitate is visible, has been advanced as an argument for the existence of two kinds of antibodies, the one a precipitin, the other a complement-binding amboceptor. Such an experiment can, in fact, be very easily carried out with any normal serum (antigen) and a good homologous antiserum. If a series of dilutions of an antiserum are prepared, a dilution can be selected which, when mixed with an appropriate quantity of the antigen, forms no precipitate but nevertheless binds complement. But if (Dean, 1912 [8]) to such a mixture of antigen and antiserum there is added fresh guinea-pig serum (complement) a precipitate does appear although a considerable interval (six to twenty-four hours) may elapse before it becomes visible. Subsequent experiments showed that the amount of the precipitate formed in such a mixture can be increased by increasing the amount of guinea-pig serum present in the mixture. Similar results were obtained if a euglobulin solution (prepared from guinea-pig serum) was substituted for ordinary normal guinea-pig serum.

Experiments of this character were carried out on numerous occasions, and as a considerable time was necessary for the formation of these precipitates, the mixtures were usually kept overnight in the

ice-chest. But if, after the precipitate has formed, the tubes are placed in an incubator, the precipitate dissolves and the mixture becomes once more absolutely clear. On replacing the tubes in the ice-chest the precipitate re-forms.

An example of such an experiment is given in Table IX.

Table IX.

No.	Fresh Guinea-pig Serum, Diluted 1 in 10.	Normal Human Serum, Diluted 1 in 500.	Anti-human Serum, Rabbit *v.* Man, Diluted 1 in 10.	Normal Saline Solution.
1	20 c.c.	0	0	1 c.c.
2	20 ,,	0·5 c.c.	0	0·5 ,,
3	20 ,,	0	0·5 c.c.	0·5 ,,
4	0	0·5 c.c.	0·5 ,,	20 ,,
5	20 ,,	0·5 ,,	0·5 ,,	0

The ingredients employed in this experiment were a 1 in 500 dilution of normal human serum, a 1 in 10 dilution of anti-human serum, and a 1 in 10 dilution of fresh guinea-pig serum. Preliminary experiments with this material had shown that equal volumes of the 1 in 500 human serum and the 1 in 10 antiserum formed a mixture which remained perfectly clear. If, however, to this mixture fresh guinea-pig serum was added, very marked fixation of complement could be demonstrated.

The amounts of each ingredient which are recorded in the table were placed in each of the five tubes. Immediately afterwards, the tubes were examined. Tube 4 was perfectly clear, while tubes 1, 2, 3, and 5 showed a similar faint degree of opalescence, which was doubtless entirely due to the normal opalescence of the guinea-pig serum. The five tubes were put in an ice-chamber and there remained for sixty hours. At the expiration of this time a large flocculent deposit had formed in tube 5. Tube 4 was absolutely clear and tubes 1, 2, and 3 showed the same faint opalescence as they had done at the beginning of the experiment. The contents of tube 5 were shaken up and all the tubes were placed in an incubator at 37° C. After two hours the precipitate had entirely disappeared from tube 5. The tubes were put back in the ice-chamber, and the precipitate re-formed in tube 5. By moving the tubes from ice-chamber to incubator, or incubator to ice-chest, the precipitate could be made to form or dissolve at will.

The experiment showed that a precipitate formed in a mixture of antigen and antibody with fresh guinea-pig serum, while in an identical mixture of antigen and antibody without the guinea-pig serum, no trace of a precipitate was visible. The aggregation of particles, which formed the precipitate, occurred at a low temperature, and the precipitate redissolved at a temperature of 37° C. The results may be explained in this fashion.

The mixture of the dilute antigen and antibody resulted in the

formation of a precipitate, the individual particles of which were so finely divided that the mixture (tube 4) appeared to be perfectly clear. Favoured by the low temperature these minute particles adsorbed euglobulin from the guinea-pig serum (tube 5) with the formation of a visible precipitate. That the euglobulin can be precipitated by a suitable mixture of antigen and antibody has already been shown (Dean, 1911 [7] and 1912 [8]).

It will be remembered that if the euglobulin is precipitated from fresh guinea-pig serum, by distilled water or weak acid, the complement is split into two fractions, the so-called mid-piece and the end-piece. The mid-piece fraction is carried down with the precipitated euglobulin while the end-piece remains in solution—Ferrata (1907 [9]), Brand (1907 [4]), Liefmann (1909 [14]), Sachs and Altmann (1909 [15]). Moreover, in complement fixation experiments the mid-piece is bound to a much greater extent than the end-piece—Skwirsky (1910 [18]), Henderson Smith (1910 [12]), Sachs and Bolkowska (1910 [16]), Amako (1910 [3]), and Gengou (1911 [10]). It may, however, well be doubted if any such substance as the mid-piece fraction of the complement exists. The action of the so-called mid-piece may be entirely due to the physical state of the adsorbed particles of euglobulin. The function of the adsorbed euglobulin would be to concentrate on the red corpuscle or bacillus the lytic substance which presumably is present in the so-called end-piece of the complement. In any case the precipitation of the euglobulin of the guinea-pig serum is an essential part of the mechanism of complement fixation. This precipitation of euglobulin is more complete at 0° C. than at 37° C., and, as Table IX. shows, the precipitate may, under certain conditions, be visible at the lower and redissolve at the higher temperature. This observation strengthens the belief that we are dealing with an adsorption process. At the lower temperature the adsorption of euglobulin by a specific precipitate is more complete. In other words antigen, antibody, and complement form a triple adsorption compound. When the antigen is a cell or bacterium susceptible to lysis, the result of this aggregation of particles is the concentration of the lytic agent, which is presumably present in the so-called end-piece fraction, at the surface of the cell which is to be dissolved. For the subsequent and quite distinct reaction which causes the solution of the cell envelope a relatively high temperature is necessary or at any rate desirable.

Summary.

1. The reagents employed were those commonly used in complement fixation reactions, namely—(*a*) Normal serum and homologous antiserum; (*b*) bacillary extract and homologous antiserum; (*c*) alcoholic organ extract and serum from a syphilitic patient.

2. The experiments showed that a mixture of antigen and anti-

body fixes more complement at 0° C. that at 37° C. Similar results were observed in the case of the Wassermann reaction.

3. In the majority of the experiments the three constituents of the reaction—the antigen, the antibody, and the complement—were brought to the required temperatures before the mixtures were prepared. The most marked differences in the results obtained at various temperatures were demonstrated in this way.

4. The time which elapses before the maximum fixation of complement occurs in any mixture of antigen and antibody depends, in the first place, on the relative proportions of antigen and antibody which are present in that mixture. If antigen and antibody are present in optimal proportions, complement is rapidly fixed. The reaction is retarded by relative excess of either antibody or antigen.

5. The maximum fixation is attained more rapidly at 37° C. than at 0° C. The amount of complement fixed is much greater at 0° C. than at 37° C.

6. The greater part of the precipitate which forms in a mixture of serum with its homologous antiserum, under the usual experimental conditions, has been shown by Chapman and Welsh to be derived from the proteids of the antiserum. From this it follows that the antiserum must not be too greatly diluted if it is desired to demonstrate the presence of a precipitate. On the other hand, antiserum can be employed in high dilution for the complement fixation reaction.

7. If fresh guinea-pig serum is added to such a mixture, containing greatly diluted antiserum and antigen, a definite precipitate is formed, although no trace of precipitation can be detected in a control tube which contains a mixture of antigen and antiserum without the addition of guinea-pig serum.

8. If suitable proportions of antigen, antiserum, and fresh guinea-pig serum are mixed, it is possible to demonstrate that a precipitate is formed at 0°C., which dissolves at 37°C. and reappears if the temperature is again brought down to 0° C.

9. When antigen, antiserum, and complement are mixed, the euglobulin of the guinea-pig serum is adsorbed by the particles of the precipitate. The formation of this adsorption compound is favoured by keeping the mixture at a low temperature. These observations explain the fact that more complement is fixed at 0° C. than at 37° C.

10. The formation of an adsorption compound in a mixture of antigen, antiserum, and guinea-pig serum, is an essential part of the mechanism of complement fixation.

11. Where the antigen is a cell which can be lysed, the effect of the formation of the adsorption compound is to concentrate at the surface of the cell the active or lytic component of the complement. The subsequent reaction which involves the lysis of the cell is, of course, favoured by a relatively high temperature.

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