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A BIOCHEMICAL STUDY OF THE PHENOMENA KNOWN AS COMPLEMENT-SPLITTING.

SECOND PAPER: SPLITTING OF THE COMPLEMENT WITHOUT A VISIBLE ALTERATION OF THE PROTEID CONSTITUENTS.*

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In the preceding communication we have dealt with various phenomena of complement-splitting in which the globulin fraction of the serum was precipitated and subsequently separated from the albumin fraction. In all these processes, according to current conceptions, the globulin fractions contained the mid-piece of complement, and the albumin fractions, the end-piece. Yet, as has already been pointed out, the so-called end-piece of the albumin portion depends in no way upon the introduction of the mid-piece in order to regain its original complement property, but can be reactivated by any reagent carrying the ions opposite in sign to those keeping the active atom-complex of complement temporarily inactive. The precipitation of the globulin fraction, therefore, was not essential in splitting the complement, but was merely an associated phenomenon. The correctness of the above deduction will be established by the experiments recorded in the following pages.

The so-called splitting of complement may be effected by certain procedures without causing the separation of the globulin fraction of complement. In one of these procedures, distilled water in certain proportions is used, and in another serum is treated with phosphates. The inactivation of complement by distilled water is doubtless due to the action upon the complement of dissociated ions of electrolytes contained in the serum, and the inactivation by a mix-

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ture of acid and alkaline phosphates (the acid being in excess) seems similar to the action of any other acid, but the presence of an alkaline phosphate modifies the reaction of the medium in such a manner that the globulin is kept in solution.

Although the phenomena of splitting complement as brought about by these chemical or physical interferences appear to be similar in mechanism to those effected by complement fixation, the complex biological reactions of the latter are difficult to analyze, and will be left for future study.

Under separate headings, we shall give the results of the other ways of splitting complement.

COMPLEMENT-SPLITTING WITH WATER.

Complement, as long as it is handled with an isotonic solution of sodium chlorid, retains its activity on dilution. On the other hand, complement rapidly loses its activity if it is diluted to a certain extent with an isotonic sugar (saccharose) solution, as was first pointed out by Sachs and Teruuchi. Thus these authors found that complement becomes completely inactive within one hour when diluted ten times with an isotonic sugar solution. The inactivation was considered by them to be due to a ferment which in a sodium chlorid-free medium manifests its activity upon complement.[1]

The above phenomenon appears, however, to be caused by some other factors than a ferment. In the case of inactivation by means of distilled water we must consider first what occurs if a solution containing two different salts of varying dissociabilities are diluted ten or more times with water. In the original concentration, the stronger electrolyte hinders the dissociation of the weaker, but when sufficiently diluted the dominating salt permits the weaker electrolyte to dissociate more fully. Sodium chlorid is a much stronger electrolyte than $NaHCO_3$ or Na_2CO_3 and it doubtless interferes with the free dissociation of the latter salts in the concentration present in serum; but when diluted ten times, its concentration no longer hinders the free dissociation of the carbonates. Thus, it becomes evident that the complement which may remain unaffected

[1] Bronfenbrenner, J., and Noguchi, H., *Jour. Exper. Med.*, 1912, xv, 598 (tables XIV and XV, A).

by the carbonates in the presence of sodium chlorid in the original concentration will be exposed to the action of the latter as soon as the sodium chlorid concentration is much less, and this is exactly what happens when serum is diluted with water. This, of course, will not occur as long as a sufficient amount of the chlorid is contained in the diluent (table I). That the change in the tonicity of the mixture (serum and diluent) has nothing to do with the loss of complement activity is shown by the fact that the use of an isotonic solution of a non-electrolyte, such as cane sugar, does not prevent the inactivation of complement.

We shall next see if the inactivation of complement by dilution with water or cane sugar solution is really an alkali inactivation. That this is the case is shown by the fact that an acid and also a few amphoteric substances may reactivate the inactive complement.[2]

TABLE I.

The Inactivation of Complement in a Salt-Free Isotonic Medium.

		Complement diluted to 40 % in 0.9 % NaCl and incubated at 37° C. for 1 hr.	Complement diluted to 40 % with 7.2 % sugar and incubated at 37° C. for 1 hr.
		0.2 c.c. of this dilution is used with varying amounts of 7.2 % sugar.	0.2 c.c. of this dilution is used with varying amounts of 0.9 % NaCl.
		Total volume made equal to 1 c.c. with 0.9 % NaCl.	Total volume made equal to 1 c.c. with 7.2 % sugar.
Varying amounts of sugar or NaCl added.	0.1	C.H.	No H.
	0.15	C.H.	Sl.H.
	0.2	C.H.	C.H.
	0.3	C.H.	C.H.
	0.4	C.H.	C.H.
	0.5	C.H.	C.H.
	0.7	C.H.	C.H.
	1 c.c.	C.H.	C.H.

COMPLEMENT-SPLITTING BY MEANS OF PHOSPHATES.

Michaelis and Skwirsky described a method by which complement can be split into end- and mid-pieces. In this method one cubic centimeter of complement (1 : 10) is mixed with two cubic centimeters of mixture A-B, which consists of sixteen parts of M/7 NaH_2PO_4 (solution A) and one part of M/7 Na_2HPO_4 (solution B), and to this are added one cubic centimeter of amboceptor (fifty

[2] Bronfenbrenner, J., and Noguchi, H., *loc. cit.*, tables XIV and XV.

TABLE II.

The Effect of Hydrochloric Acid upon the Inactivation of Complement by Distilled Water.

		0.5 c.c.	0.4 c.c.	0.3 c.c.	0.2 c.c.	0.15 c.c.	0.1 c.c.	0.07 c.c.	0.05 c.c.	0.04 c.c.	0.03 c.c.	0.02 c.c.
Native complement + 0.9% NaCl added after 15 min.	1.0 c.c. 9.0 c.c.								C.H.	F.C.H.	Sl.H.	No H.
Complement + distilled water + 10% NaCl added after 15 min.	1.0 c.c. 8.2 c.c. 0.8 c.c.						C.H.	F.C.H.	Sl.H.	Tr.H.	No H.	
Complement + distilled water + 10% NaCl added after 30 min.	1.0 c.c. 8.2 c.c. 0.8 c.c.	No H.										
Complement HCl N/250 + NaOH N/25 in 10% NaCl added after 15 min.	1.0 c.c. 8.2 c.c. 0.8 c.c.		C.H.	F.C.H.	Sl.H.	Sl.H.	No H.					
Complement HCl N/250 + NaOH N/25 in 10% NaCl added after 30 min.	1.0 c.c. 8.2 c.c. 0.8 c.c.		C.H.	F.C.H.	Sl.H.	Sl.H.	No H.					
Complement HCl N/250 + NaOH N/25 in 10% NaCl added after 2 hrs.	1.0 c.c. 8.2 c.c. 0.8 c.c.		C.H.	F.C.H.	Sl.H.	Tr.H.	No H.					
Complement + 0.9% NaCl after 2 hrs. (control).	1.0 c.c. 9.0 c.c.								C.H.	Mch.H.		

units) and one cubic centimeter of 5 per cent. sheep corpuscles. After incubation for one hour at 37° C. the mixture is centrifugalized and the supernatant fluid is separated from the deposited corpuscles. To the supernatant fluid, two cubic centimeters of solution B are added and the whole is called the end-piece. The corpuscles are suspended in salt solution and are used as persensitized corpuscles (sensitized and carrying the mid-piece of complement). When the persensitized corpuscles are mixed with the end-piece they undergo hemolysis, while the sensitized corpuscles are not acted upon by the end-piece.

The above phenomenon is probably explained by the fact that complement is highly sensitive to the action of various acids, alkalis, and salts, and undergoes a reversible inactivation. It is not at all improbable, therefore, that the mixture of solutions A and B, which is strongly acid to phenolphthalein, renders the complement inactive, while the persensitization goes on without hinderance in the same medium. The acidity of the supernatant fluid is, in this instance, to a certain degree reduced by the later addition of solution B so that the inactivated complement, which is near the border of rever-

TABLE III.

Reaction and Anticomplementary Activity of Different Mixtures of Phosphates.

	Reaction of 1 c.c.		Anticomplementary activity upon 0.04 c.c. of complement + 2 units of amboceptor.	
	Indigo-alizarin.	Phenolphthalein.	Complete hemolytic dose.	Non-hemolytic dose.
Solution A. 27.4 gm. NaH_2PO_4 per 1,000 c.c.	Acid +0.4 c.c. NaOH N/100	Acid 24 c.c. NaOH N/100	0.07 c.c.	0.2 c.c.
Solution B. 51.4 gm. Na_2HPO_4 per 1,000 c.c.	Alkali 15 c.c. HCl N/100	Alkali 0.7 c.c. HCl N/100	0.5 c.c.	1 c.c.
Mixture A−B. 16 c.c. A+1 c.c. B.	Alkali 0.45 c.c. HCl N/100	Acid 22 c.c. NaOH N/100	0.1 c.c.	0.25 c.c.
Neutralized Mixture. 1 c.c.A-B+1 c.c. B.			0.5 c.c.	1 c.c.

sion, regains its activity whenever an adequate quantity of certain alkali (table IV) or amphoteric compounds is introduced (table V).

TABLE IV.

Reactivation of Complement Inactivated by Phosphates.

	NaOH N/1 in c.c.									
	0.1	0.07	0.05	0.03	0.02	0.01	0.005	0.001	0.0007	0.0005
Complement[3] 0.04 c.c. + 27.4/1,000 NaH_2PO_4 0.2 c.c. in each tube + 3 units of amboceptor.	Alkali hemolysis.			C.H.	C.H.	C.H.	C.H.	No H.	No H.	No H.
	Zone neutral to phenolphthalein.							Zone neutral to alizarin-indigo-carmin.		

	HCl N/1 in c.c.										
	0.2	0.1	0.07	0.05	0.03	0.0275	0.025	0.02	0.01	0.007	0.005
Complement[3] 0.04 c.c. + 51.4/1,000 Na_2HPO_4 1 c.c. in each tube + 3 units of amboceptor.	Acid hemolysis.		No H.	Sl.H.	C.H.	Sl.H.	No H.	No H.	No H.	No H.	No H.
	Zone neutral to phenolphthalein.								Zone neutral to alizarin-indigo-carmin.		

From the foregoing experiments it will be seen that the inactivated complement is to be found in the supernatant fluid in a reversible state. The reversion is accomplished by relieving the complement of the acid reaction by any suitable substance. The reason that the corpuscles digested in the Michaelis-Skwirsky mixture are capable of reversing the inactivated complement may be due to the absorption of a certain amount of serum constituents (probably the globulin fraction), also the absorption of the alkaline phosphate by certain constituents of the cells. It appears from the following experiments that the presence of amboceptor has a certain influence in enabling the corpuscles to absorb them, because without the amboceptor the persensitization is seen to be less complete (table VI).

That the end-piece of complement as obtained by the method of Michaelis and Skwirsky is in a general way similar to those obtained by other processes is well shown in the following experiments. It is most striking that alanin plays beautifully the rôle of the midpiece with all end-pieces here studied (table VII, C).

[3] The complement was incubated with the phosphates at 37° C. for 30 minutes, then alkali and acid, respectively, were added and the whole was reincubated for 30 minutes.

TABLE V.

Reactivation of Complement Inactivated by Phosphates.

Complement 0.04 c.c. + Na_2HPO_4 (solution 51.4gm./1,000) 1 c.c. in each tube incubated for 30 minutes at 37° C. Then varying amounts of reactivating substances were added, the volume brought to 2 c.c. in each tube and incubated for 30 minutes at 37° C.; finally 10 units of amboceptor and 0.1 c.c. of a 10% suspension of corpuscles were added and again incubated for 30 minutes.											Na_2HPO_4. (No complement.)	
	0.05 c.c.	0.1 c.c.	0.2 c.c.	0.3 c.c.	0.4 c.c.	0.5 c.c.	0.6 c.c.	0.7 c.c.	1 c.c.	Control.	0.5 c.c.	1 c.c.
Egg-white (1:5)					No H.	Sl.H.	F.C.H.	C.H.	C.H.			No H.
Serum 56° C. (sheep) ...			No H.	Sl.H.	Mch.H.	C.H.						No H.
Alanin 3% in NaCl (0.9%)	No H.	Sl.H.	F.C.H.	C.H.								No H.
HCl N/10 in 0.9% NaCl.	No H.	F.C.H.	C.H.	C.H.	Sl.H.	No H.					No H.	C.H.
0 No reactivating substances										No H.		

Same as above except that 0.2 c.c. NaH_2PO_4 (solution 27.4/1,000) was used instead of 1 c.c. of Na_2HPO_4.											NaH_2PO_4. (No complement.)	
	0.05 c.c.	0.1 c.c.	0.2 c.c.	0.3 c.c.	0.4 c.c.	0.5 c.c.	0.6 c.c.	0.7 c.c.	1 c.c.	Control.	0.3	0.5
Egg-white (1:5)				No H.	Sl. H.	F.C.H.	Sl.H.	No H.				
Serum 56° C. (sheep) ...	Tr.H.	Sl.H.	C.H.	C.H.	C.H.	F.C.H.	No H.					
Alanin 3% in 0.9% NaCl						No H.	No H.	Sl.H.	F.C.H.			
NaOH N/10 in 0.9% NaCl	F.C.H.	C.H.	C.H.	C.H.	F.C.H.	Sl.H.					No H.	C.H.
0 No reactivating substances										No H.		

TABLE VI.

Can Corpuscles be Persensitized without Amboceptor, or Complement, or Both?

Tubes.	1	2	3	4	5	6
	Complement (1:10) 0.2 c.c. Mixture A-B 0.4 c.c. Amboceptor (20 units) 0.2 c.c. Corpuscles (5%) 0.2 c.c.	Heated complement (1:10) 0.2 c.c. Mixture A-B 0.4 c.c. Amboceptor (20 units) 0.2 c.c. Corpuscles (5%) 0.2 c.c.	0.9% NaCl 0.2 c.c. Mixture A-B 0.4 c.c. Amboceptor (20 units) 0.2 c.c. Corpuscles (5%) 0.2 c.c.	Complement (1:10) 0.2 c.c. Mixture A-B 0.4 c.c. NaCl 0.9% 0.2 c.c. Corpuscles (5%) 0.2 c.c.	Complement (1:10) 0.2 c.c. Mixture A-B 0.4 c.c. Amboceptor (20 units) 0.2 c.c. NaCl (0.9%) 0.2 c.c.	Complement (1:10) 0.2 c.c. Mixture A-B 0 Amboceptor 0 Corpuscles (5%) 0.2 c.c. NaCl (0.9%) 0.6 c.c.
Remarks		Heated complement	No complement	No amboceptor	No corpuscles	No mixture A-B No amboceptor
All mixtures incubated for 60 minutes at 37° C. and corpuscles separated by centrifugalization (to be used later).						
0.4 c.c. of solution B (Na_2HPO_4) added to all the supernatant fluids except the last (6).						0.4 c.c. NaCl added.
To the different amounts of the supernatant fluid of each tube the corpuscles of each of the other tubes were added and again incubated; results taken after 1 hour.						

(Continued on page 633.)

TABLE VI (*Continued*).

Corpuscles from tubes.	Supernatant fluid from tube 1.				Supernatant fluid from tube 2.			Supernatant fluid from tube 3.		
	0.7 c.c.	0.5 c.c.	0.3 c.c.	0.2 c.c.	1 c.c.	0.7 c.c.	0.5 c.c.	1 c.c.	0.7 c.c.	0.5 c.c.
1	C.H.	F.C.H.	Sl.H.	No H.	No H.			No H.		
2	C.H.	F.C.H.	Tr.H.	No H.	No H.			No H.		
3	C.H.	Mch.H.	No H.		No H.			No H.?		
4	F.C.H.	Sl.H.	No H.		No H.			No H.		
6	F.C.H.	Sl.H.	No H.		No H.			No H.		
Non-treated corpuscles (5%) 0.2 c.c.	F.C.H.	Sl.H.	No H.		No H.			No H.		

Corpuscles from tubes.	Supernatant fluid from tube 4.				Supernatant fluid from tube 5.				Supernatant fluid from tube 6.		
	0.7 c.c.	0.5 c.c.	0.3 c.c.	0.2 c.c.	0.7 c.c.	0.5 c.c.	0.3 c.c.	0.2 c.c.	0.3 c.c.	0.2 c.c.	0.15 c.c.
1	C.H.	F.C.H.	Tr.H.	No H.	C.H.	Sl.H.	No H.		C.H.	Mch.H.	No H.
2	C.H.	Mch.H.	No H.		C.H.	Mch.H.	No H.		C.H.	Mch.H.	No H.
3	C.H.	Mch.H.	Tr.H.	No H.	C.H.	Mch.H.	No H.		C.H.	Sl.H.	No H.
4	C.H.	Sl.H.	No H.		C.H.?	No H.	No H.		C.H.	Mch.H.	No H.
6	C.H.	Mch.H.	Tr.H.?	No H.	F.C.H.	Mch.H.	No H.		C.H.	Sl.H.	No H.
Non-treated corpuscles (5%) 0.2 c.c.	C.H.	Sl.H.	No H.		F.C.H.	Mch.H.	No H.		C.H.	Mch.H.	No H.
	Amboceptor (20 units) 0.2 c.c. added.								Amboceptor (20 units) 0.2 c.c. added.		

TABLE VII.

Activity of Different End-Pieces with Various Mid-Pieces and Alanin.

	0.5 c.c. of 5 % suspension of fresh corpuscles + 50 units of amboceptor.		Same amount of persensitized corpuscles.		Same amount of sensitized corpuscles.		0.5 c.c. of 5 % fresh corpuscles + 50 units of amboceptor + 0.5 c.c. of mid-piece (CO_2).		0.5 c.c. of 5 % fresh corpuscles + 50 units of amboceptor + 0.5 c.c. HCl mid-piece.		0.5 c.c. of 5 % fresh corpuscles + 50 units of amboceptor + 0.3 c.c. of 3 % alanin.	
Amounts of end-piece used.	0.5 c.c.	0.3 c.c.	0.5 c.c.	0.3 c.c.	0.5 c.c.	0.3 c.c.	0.5 c.c.	0.3 c.c.	0.5 c.c.	0.3 c.c.	0.5 c.c.	0.3 c.c.
(A) End-piece (CO_2) made isotonic.	No H.	No H.	C.H.	C.H.	F.C.H.	C.H.	Mch.H.	No H.	C.H.	C.H.	C.H.	C.H.
(B) End-piece (HCl) made neutral and isotonic.	No H.	No H.	C.H.	C.H.	Sl.H.(?)	No H.	F.C.H.	Sl.H.	C.H.	C.H.	Sl.H.	No H.
Amounts of Skwirsky's[4] end-piece used.	2.5 c.c.	1.5 c.c.	2.5 c.c.	1.5 c.c.	2.5 c.c.	1.5 c.c.	2.5 c.c.	1.5 c.c.	2.5 c.c.	1.5 c.c.	2.5 c.c.	1.5 c.c.
(C) Skwirsky's end-piece neutralized by solution B.	No H.	No H.	C.H.	C.H.	Sl.H.(?)	No H.	C.H.	Sl.H.	C.H.	F.C.H.	C.H.	Sl.H.

[4] Skwirsky's end-piece being more diluted, it was necessary to increase the quantity accordingly.

COMPLEMENT-SPLITTING IN COMPLEMENT DEVIATION PHENOMENA.

Much more complicated is the phenomenon first described by Skwirsky, who found that the supernatant fluid of the mixture containing a positive syphilitic serum, syphilitic antigen, complement, sheep's corpuscles, and antisheep amboceptor, has the power to cause hemolysis upon a fresh lot of sheep corpuscles when the latter are previously persensitized. By the term of persensitization is meant the digestion of washed sheep corpuscles in a mixture of sixteen

TABLE VIII.

Action of Mixture A-B upon the Substances Concerned in the Wassermann Reaction.

1

Mixture	Amount
Syphilitic serum (heated at 56° C.)	0.1 c.c.
Antigen (0.3 %) (lipoids)	0.5 c.c.
Complement (1:10)	0.5 c.c.
NaCl (0.9 %)	0.4 c.c.
	1.5 c.c.

Each of the tubes contained this mixture (1.5 c.c.) The tubes were incubated at 37° C. for 30 min. then varying amounts of A–B mixture were added and the volume was brought up to 2.5 c.c. with NaCl (0.9 %). After the second incubation for 30 min. at 37° C., 0.5 c.c. of 5 % sheep corpuscles and 50 units of amboceptor were added and the whole was again incubated for 30 min. at 37° C.

Varying amounts of the mixture A–B.	1 c.c.	0.7 c.c.	0.5 c.c.	0.3 c.c.	0.2 c.c.	0.1 c.c.	0.07 c.c.	0.05 c.c.	0
Result of the hemolytic test.	No H.	No H.	No H.	No H.	No H.	No H.	No H.	No H.	No H.

2

In this series, corpuscles were digested with amboceptor and mixture A–B for 30 min. at 37° C., and then to each tube 1.5 c.c. of the above mixture of complement, antigen, syphilitic serum, and NaCl were added. The amounts of substances used were the same as above.

Varying amounts of mixture A–B + corpuscles 5 % 0.5 c.c. + amboceptor 50 units.	1 c.c.	0.7 c.c.	0.5 c.c.	0.3 c.c.	0.2 c.c.	0.1 c.c.	0.07 c.c.	0.05 c.c.	0
Results of the hemolytic test after additions of 1.5 c.c. of the above mixture (1).	Sl.H.	Sl.H.	Sl.H.	Sl.H.	Sl.H.	Sl.H.	Sl.H.	Tr.H.	No H.

3

Corpuscles (0.5 c.c. of 5 % suspension) were digested for 30 min. at 37° C. with complement (1:10) 0.5 c.c. + amboceptor (50 units) 0.5 c.c. + mixture A–B 1 c.c.; then the corpuscles were separated by centrifugalization and acted upon by varying amounts of mixture 1.

Amounts of mixture 1 added; total volume made equal to 3.5 c.c.	1 c.c.	0.7 c.c.	0.5 c.c.	0.3 c.c.	0.2 c.c.	0.1 c.c.	0
Results of the hemolytic experiment.	C. H.	C. H.	C. H.	Sl. H.	No H. (?)	No H.	No H.

parts of M/7 solution of NaH_2PO_4 and one part of M/7 solution of Na_2HPO_4, to which a definite amount of complement and a large quantity of antisheep amboceptor have also been added (table IX, 2). The corpuscles digested in this mixture are hemolyzable by the supernatant fluid separated from a positive fixation test. The ordinary corpuscles with or without amboceptor are not hemolyzed by the supernatant fluid. Thus, this phenomenon was explained by them as being due to the presence of the end-piece of the complement in the supernatant fluid which, by virtue of the absorption of the mid-piece of complement by the sensitized corpuscles in the digestion procedure, becomes active upon the latter.

This phenomenon was confirmed by various investigators and we have also obtained a similar result (table IX).

Curiously enough, however, this apparent splitting of complement during the fixation by the Wassermann or Bordet-Gengou reaction does not take place when the antihuman hemolytic system is used (table X).

It is also interesting to note that the supernatant fluid of the Wassermann reaction which is quite active for the persensitized sheep corpuscles is almost inactive upon fresh sheep corpuscles when varying amounts of the mixture of solution A-B and fifty units of amboceptor are added (table XI, B). Apparently the addition of the A-B mixture and amboceptor alone is not sufficient to make the Wassermann supernatant fluid active. On the other hand, when the mixture A-B is allowed to act previously upon the sheep corpuscles with fifty units of antisheep amboceptor, the Wassermann supernatant fluid shows a certain amount of hemolytic effect. Here it seems as if the A-B mixture had so altered the corpuscles that they became to a certain extent hemolyzable by the Wassermann supernatant fluid (table XI).

It was also found that the persensitization of sheep corpuscles takes place in a hypertonic sodium chlorid or sugar solution, as indicated in the following experiments (table XII). At the same time it is interesting to notice that the Wassermann supernatant fluid is inactive upon the corpuscles persensitized when the medium is hypertonic.

The heating of the Wassermann supernatant fluid to 56° C. for

TABLE IX.

Splitting of Complement in the Wassermann Reaction with the Antisheep Hemolytic System.

1 Designated as Wassermann supernatant fluid.	2 Designated as persensitized corpuscles.	3 Designated as sensitized corpuscles.
Syphilitic serum (56° C.) 0.1 c.c. Antigen (lipoids) (0.3 %) 0.5 c.c. Complement (1:15) 0.5 c.c. NaCl (0.9 %) 0.4 c.c.	Mixture A-B 1 c.c. Corpuscles (5 %) 0.5 c.c. Amboceptor (50 units) 0.5 c.c. Complement (1:10) 0.5 c.c.	Suspension of corpuscles (10 %) 2.5 c.c. Amboceptor (50 units) 2.5 c.c.
Incubated 30 min. at 37° C.	Incubated 60 min. at 37° C. Then corpuscles were separated by centrifugalization and resuspended in 0.5 c.c. of 0.9 % NaCl.	Incubated 60 min. at 37° C. Then corpuscles were separated by centrifugalization and resuspended in 0.5 c.c. of 0.9 % NaCl.

Wassermann supernatant fluid (0.5 c.c. of complement 1:15 diluted in 1.5 c.c. of fluid).					Complement (1:15) 0.5 c.c. + NaCl (0.9 %) 1 c.c. (0.5 c.c. of complement 1:15 diluted in 1.5 c.c. of salt solution).				
Amounts of Wassermann supernatant fluid used.	+ 0.5 c.c. of persensitized corpuscles.	+ 0.5 c.c. of sensitized corpuscles. + 0	+ 0.5 c.c. of sensitized corpuscles. + Mid-piece[5] (CO_2) 1:1	+ 0.5 c.c. of sensitized corpuscles. + Alanin 3 % 0.3 c.c.	Amounts of dilution of complement used.	+ 0.5 c.c. of persensitized corpuscles.	+ 0.5 c. of sensitized corpuscles. + 0	+ 0.5 c. of sensitized corpuscles. + Mid-piece[5] (CO_2) 1:1	+ 0.5 c. of sensitized corpuscles. + Alanin 3 % 0.3 c.c.
1.0 c.c.	C.H.	No H.	No H.	No H.	1.0 c.c.	C.H.	C.H.		
0.7 c.c.	C.H.				0.7 c.c.	C.H.	C.H.		
0.5 c.c.	Sl.H.				0.5 c.c.	Sl.H.	C.H.		
0.3 c.c.	No H.				0.3 c.c.	No H.	F.C.H.	C.H.	C.H.
0.2 c.c.					0.2 c.c.		Sl.H.	Mch.H.	Sl.H.
					0.15 c.c.		No H.	No H.	No H.

[5] The amount of mid-piece used was equal to the amount of complement present in the corresponding amount of dilution used; namely, 1 c.c. of complement was broken up by CO_2 and the globulin fraction, collected by centrifugalization, dissolved in 15 c.c. of NaCl and 0.3 c.c., 0.2 c.c., 0.15 c.c., 0.07 c.c., respectively, were taken for 1 c.c., 0.7 c.c., 0.5 c.c., 0.3 c.c., of the Wassermann supernatant fluid.

TABLE X.

The Presence of End-Piece in the Supernatant Fluid of the Wassermann and Bordet-Gengou Reactions Tested with the Antihuman Hemolytic System.[6]

Designated as Wassermann supernatant fluid (comparable to table IX, 1).

Syphilitic serum (56° C.)	0.1 c.c.
Antigen (0.3 %)	0.5 c.c.
Complement (1:15)	0.5 c.c.
NaCl (0.9 %)	0.4 c.c.

Incubated 30 min. at 37° C.

	Mid-piece (CO_2).		No mid-piece (control).
	0.04 c.c.	0.02 c.c.	
The above Wassermann[7] supernatant fluid 1.5 c.c.			
End-piece (CO_2) 0.04 c.c.	No H.	No H.	No H.
0 (control).	C.H. No H.	C.H.	No H.
	+ 0.5 c.c. of persensitized corpuscles.		
Wassermann supernatant fluid 1.5 c.c.	No H.		

Designated as mixture II.

Egg-white (1:10)	1 c.c.
Anti-egg serum (1:10)	1.5 c.c.
NaCl (0.9 %)	7.5 c.c.

Incubated 60 min. at 37° C., then shaken up and varying amounts incubated again with 0.1 c.c. of 40 % complement.

	Amount of mixture II added to 0.1 c.c. of 40 % complement.				
	0	0.2 c.c.	0.4 c.c.	0.7 c.c.	1 c.c.
0.5 c.c. of 5 % fresh corpuscles + 50 units of amboceptor	C.H.	C.H.	C.H.	Sl. H.	No H.
0.5 c.c. of persensitized corpuscles.	C.H.	F.C.H.	Sl. H.	No H.	

Designated as supernatant fluid III.

Sheep serum (56° C.)	0.1 c.c.
Antisheep serum (56° C.)	0.1 c.c.
Complement (40 %)	0.1 c.c.
NaCl (0.9 %)	0.7 c.c.

Incubated 60 min. at 37° C., centrifugalized and supernatant fluid examined.

	+ 0.2 c.c. mid-piece.	No mid-piece.
Supernatant fluid III 1 c.c.	No H.	No H.
End-piece (CO_2) 0.04 c.c.	C.H.	No H.
	+ 0.5 c.c. of persensitized corpuscles.	
Supernatant fluid 1 c.c.	No H.	
Native complement 0.04 c.c.	C.H.	
End-piece (CO_2) 0.04 c.c.	C.H.	

[6] The table is arranged to show the three different procedures used in each of these experiments, all of which show the same result, contrary to the experiments with antisheep hemolytic system.

[7] In this experiment the human hemolytic system was used and in all the tubes the total volume was brought to 2 c.c. and when necessary 50 units of amboceptor and 0.5 c.c. of a 5 % suspension of human corpuscles were added.

TABLE XI.

The Effect of Mixture A-B upon Sheep Corpuscles and Wassermann Supernatant Fluid, Respectively.

		Amounts of solution A-B added.							0.5 c.c. of persensitized corpuscles. Controls.	0.5 c.c. of sensitized corpuscles. Controls.
A	Wassermann[8] supernatant fluid 1.5 c.c. added to all. After additions of A-B, incubated at 37° C. for 30 min. 0.5 c.c. of 5% sheep corpuscles and 50 units amboceptor. Incubated for 60 min., and results taken.	1 c.c.	0.7 c.c.	0.5 c.c.	0.1 c.c.	0.5 c.c.	0.03 c.c.	0.01 c.c.	0	0
		No H.	No H.	No H.	No H.	No H.	No H.	No H.	C.H.	No H.

		Amounts of solution A-B added.								Controls	Controls
B	Corpuscles (5%) 0.5 c.c. Amboceptor (50 units) 0.5 c.c. Varying amounts of solution A-B. Incubated at 37° C., for 30 min., then[8] 1.5 c.c. Wassermann supernatant fluid added to each tube and after incubation for 60 min., results taken.	1 c.c.	0.7 c.c.	0.5 c.c.	0.3 c.c.	0.2 c.c.	0.1 c.c.	0.05 c.c.	0.03 c.c.	0	0
		No H.(?)	Sl.H.(?)	Sl.H.	Sl.H.	Sl.H.	Sl.H.	Tr.H.	No H.	C.H.	No H.

[8] See table IX, mixture I.

TABLE XII.

Does Persensitization Occur in a Hypertonic Medium?

<table>
<tr><td colspan="4">Complement (1:15) 0.5 c.c.
Amboceptor 50 units 0.5 c.c.
Mixture A-B 1.0 c.c.
Sheep corpuscles 5% 0.5 c.c.
0.9% NaCl solution 0.5 c.c.</td><td colspan="2">Complement (1:15) 0.5 c.c.
Amboceptor 50 units 0.5 c.c.
Mixture A-B 1.0 c.c.
Sheep corpuscles 5% 0.5 c.c.
10% NaCl solution 0.5 c.c.</td><td colspan="2">Complement (1:15) 0.5 c.c.
Amboceptor (50 units) 0.5 c.c.
Mixture A-B 1.0 c.c.
Sheep corpuscles (5%) 0.5 c.c.
10 times isotonic (72%) sugar solution 0.5 c.c.</td></tr>
<tr><td colspan="4">After 30 min. incubation at 37° C., corpuscles separated and used for the following experiment.</td><td colspan="2">After 30 min. incubation at 37° C., corpuscles separated and used for the following experiment.</td><td colspan="2">After 30 min. incubation at 37° C., corpuscles separated and used for the following experiment.</td></tr>
<tr><td colspan="3">1.5 c.c. Wassermann supernatant fluid</td><td rowspan="2">1.5 c.c. of 0.9% NaCl +0.5 c.c. of 0.9% NaCl</td><td rowspan="2">1.5 c.c. of 0.9% NaCl +0.5 c.c. of 0.9% NaCl</td><td rowspan="2">1.5 c.c. of Wassermann supernatant fluid +0.5 c.c. of 0.9% NaCl</td><td rowspan="2">1.5 c.c. of 0.9% NaCl +0.5 c.c. of 0.9% NaCl</td><td rowspan="2">1.5 c.c. of Wassermann supernatant fluid +0.5 c.c. of 0.9% NaCl</td></tr>
<tr><td>+0</td><td>+0.5 c.c. of 10% NaCl</td><td>+0.5 c.c. of 72% Sugar</td></tr>
<tr><td>C.H.</td><td>No H.</td><td>F.C.H.</td><td>No H.</td><td>No H.</td><td>C.H.</td><td>No H.</td><td>C.H.</td></tr>
</table>

TABLE XIII.

The Activation of Different End-Pieces by Different Mid-Pieces and Alanin in the Presence of Sheep or Human Corpuscles.

		End-piece (CO_2) isotonic, 0.4 c.c.		End-piece (HCl) neutralized and isotonic, 0.4 c.c.		Wassermann supernatant fluid, 1.5 c.c.		End-piece (Skwirsky)[9] neutralized by B, 3.5 c.c.		0.9% NaCl, 3.5 c.c.	
		Corpuscles.		Corpuscles.		Corpuscles.		Corpuscles.		Corpuscles.	
		Human.	Sheep.	Human.	Sheep.	Human.	Sheep.	Human.	Sheep.	Human.	Sheep.
Mid-piece (CO_2) 0.2 c.c.	50 units of amboceptor and 0.5 c.c. of 5% puscles	Sl.H.	Mch.H.	Sl.H.	C.H.	No H.	No H.	No H.	F.C.H.	No H.	No H.
Mid-piece (HCl) 0.2 c.c.		Mch.H.	F.C.H.	Mch.H.	C.H.	No H.	No H.	No H.	F.C.H.	No H.	No H.
Alanin (3%) 0.3 c.c.		Mch.H.	Mch.H.	C.H.	C.H.	No H.	No H.	No H.	No H.	No H.	No H.
Mid-piece of heated complement (CO_2) 0.2 c.c.		Mch.H.	Mch.H.	Sl.H.	Sl.H.	No H.	No H.	No H.	Sl.H.	No H.	No H.
0.5 c.c. of persensitized corpuscles.		Sl.H.	C.H.	Sl.H.	C.H.	No H.	F.C.H.	F.C.H.	C.H.	No H.	No H.

[9] Skwirsky's end-piece is the supernatant fluid (described in table IX, 2) resulting after persensitized corpuscles are removed from the mixture of A-B + complement + amboceptor + corpuscles. This supernatant fluid (2.5 c.c.) is to be neutralized by 1 c.c. of solution B and the result is designated Skwirsky's end-piece.

thirty minutes destroys its activity upon persensitized sheep corpuscles. The supernatant fluid from the positive Wassermann serum in which an inactivated complement, instead of fresh complement, had been used, is totally inactive upon the persensitized corpuscles, while the persensitization of the corpuscles can be accomplished by means of an inactivated as well as an active complement (table VI, 2).

From the fact that the sheep corpuscles separated from a positive Wassermann or Bordet-Gengou fixation test remain sensitized, but not persensitized, it is evident that the mid-piece in this instance interacts with the mixture of syphilitic serum and lipoids or antibody and antigen, thus becoming incapable of persensitizing the sensitized corpuscles. Just what kind of interaction has taken place is at present undetermined, but one point is certain and that is that the mid-piece is completely used up.

In this respect we may cite here an interesting phenomenon already observed by Noguchi and Bronfenbrenner (1910): namely, the interference exerted by certain amphoteric substances (egg-white, inactivated sera, etc.) upon the fixation phenomenon. Apparently the fixing molecules of the antibody-antigen combination are saturated by these substances which in this respect resemble the mid-piece.

GENERAL CONSIDERATION.

Thus far, most investigators have made but little distinction between the splitting phenomenon obtained by chemical interference and that which takes place in the biological phenomenon known as complement fixation. In this study we have shown that these two sets of phenomena have certain fundamental differences and that the so-called complement-splitting by physical conditions leading to chemical interaction, or directly by chemical means, is not a real splitting of the complement, but an inactivation of the active principle of complement through an alteration in the reaction of the medium caused by an excess of either anions or kations. The modification of the reaction of the medium may cause a more or less definite combination of the complement with the free ions, but the latter can readily be removed by an appropriate number of opposite

ions, and render the complement active once more. The fluids that have hitherto been regarded as containing the end-piece of complement, as a matter of fact, contain the whole complement temporarily deprived of its activity by certain ions derived either from the salt constituents of the serum itself under a modified physical condition (dialysis against water or dilution with water) or introduced in the form of dissociable electrolytes.

On the other hand, the splitting of complement in the fixation reaction seems far more complicated than that caused by the physical or chemical procedures. The supernatant fluid from the fixation test differs from all the other end-pieces prepared by chemical methods in being active upon persensitized sheep corpuscles only (not upon human corpuscles). The addition of various mid-pieces obtained by different methods to sensitized sheep corpuscles does not render the Wassermann supernatant fluid active (table XIII). It is quite remarkable that the persensitized sheep corpuscles are, on the other hand, easily attacked, not only by the supernatant fluids of fixation tests, but also equally well by the other end-pieces. It is not at all improbable that in the fixation reaction, where so many factors come into play, there is a most complicated physical as well as chemical interaction leading to such an entangled mixture of factors that a substance carrying one set of ions alone cannot reverse the activity of complement, and hence the reversion takes place only when certain electrolytes with both ions are employed. At all events there seems to be no doubt that the inactivation of complement is far more complicated in the Wassermann reaction or the Bordet-Gengou phenomenon than in the inactivation by physical or chemical means. Nevertheless, no one has as yet proved conclusively that the supernatant fluid of a fixation test necessarily contains the end-piece of complement.