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STUDIES ON THE INACTIVATION OF COMPLEMENT BY SHAKING.

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INTRODUCTION.

IN a former paper on the inactivation of the haemolytic complement by means of shaking (1913), I considered it possible that the process of shaking-inactivation might be explained by assuming that an adsorption of the complement occurred on the surfaces of the precipitate, which is always found when a serum is shaken. I was unable however either to fix the complement of a fresh serum by adding the centrifuged coagula of a shaken serum or, on the other hand, to set free a complement presumably adsorbed on the surface of precipitated proteins, which are found to be soluble only in alkali. I therefore had to leave undecided the question as regards the nature of the process of shaking-inactivation.

About the same time and independently P. Schmidt and M. Liebers (1913), working on the shaking-inactivation of the haemolytic complement, came to the following conclusion: shaking of guinea-pig serum produces precipitation of the euglobulins followed by adsorption of the complement on these newly-formed surfaces. The visibly coagulated globulin is only a minimal part of the total globulin, and the shaken and centrifuged serum contains therefore not only globulins but also complement not yet adsorbed. They succeeded in bringing the globulin, precipitated by shaking, back into solution by a normal E-piece, thus setting free the complement adsorbed on these surfaces, enabling it to act again.

L. Hirschfeld and R. Klinger (1914), in their researches on the shaking-inactivation, worked with human serum and came to the conclusion that shaking produces alteration in the stability of the globulins, thus causing a part of them to coagulate. This alteration interferes

with complement action, and sera so treated become anti-complementary, giving for instance a positive Wassermann reaction. Recent work of A. Bessemans (1913), however, shows that human and guinea-pig sera are different as regards the stability of their globulins, and results, obtained with experiments on human serum, are therefore not to be compared with those obtained with guinea-pig serum except to a restricted degree.

I therefore resumed my investigations on the nature of the inactivation process by shaking chiefly with a view to ascertain in how far the conception of P. Schmidt could be adapted to the experimental facts.

I give below a record of experiments made from a serological point of view as regards the influence of shaking upon the haemolytic complement of guinea-pig serum, and consider them to be a continuation of my former work on the same subject.

Technique of experiments.

The technique employed in shaking the sera is principally the same as that indicated in my former paper (1913). Haemolysis was due to the complementing action of the serum in combination with sensitized sheep red corpuscles, of which a 2·5 % emulsion in 0·85 % saline was used. Sensitization was effected with inactivated rabbit immune-serum, the dose of which will be given in the protocols. The following scheme has been adopted to illustrate different degrees of haemolysis:

- No haemolysis.
- Faint trace of haemolysis.
- Very slight haemolysis.
- Well-marked haemolysis.
- Half haemolysed.
- Strong haemolysis.
- Very strong haemolysis.
- Nearly complete haemolysis [a slight trace of unlaked cells at the bottom].
- Complete haemolysis.

Experimental part.

In another communication (1914), considering the effect of shaking upon a serum from a more general standpoint, I have shown that the effect of shaking upon a serum is due to alterations in the surface energy

involving changes of the serum albumins and denaturation of the serum proteins, which first of all affects the euglobulin. The process is to be considered as progressing with the time, till irreversible denaturation of the proteins is effected. The various stages of this process are serologically characterised by the steadily progressing loss of complement activity as indicated by the different procedures necessary to restore the complementing power. The different factors necessary to the restitution of complement activity have been already mentioned in my former paper (1913), but before I can attempt to explain these very complicated relations, I must first of all complete the description of my experiments.

Reactivation of a shaken serum by a fresh serum.

A serum inactivated by shaking can be reactivated by the addition of fresh serum. This reactivation can be effected under any conditions, and it is the better marked the more the shaken inactivated serum can be reactivated by other factors, as will later be shown. The question as to the restitution of a shaken serum by a normal serum is, in reality, how far the former interferes with the action of normal complement. Jakoby and Schuetze (1910) found no inhibition to take place, a fact which I can confirm. It proved to be the same, whether the precipitated globulins were removed by the centrifuge and filtering, or not removed at all. I found also that the precipitate itself showed no inhibiting effect on normal complement action. Only when present in large excess did slight inhibition sometimes occur. This is confirmed by the work of Landsteiner and Stankovic (1906), who found that heat-coagulated serum proteins produced a slight inhibiting effect. Hirschfeld and Klinger (1914), however, found that sometimes the centrifuged precipitate of a shaken serum exerted an anti-complementary effect, but this was not constant. On the other hand the sera after shaking were sometimes so anti-complementary as to give a positive Wa. reaction, even if the serum had been well centrifuged. But it must be remembered that these authors worked exclusively with human serum, which may behave very differently from guinea-pig serum when shaken, the latter showing no inhibiting effect after shaking, as also Courmont and Dufourt found (1912).

Whether inhibition of normal complement action takes place or not would according to P. Schmidt's conception be explained by assuming a partial or complete saturation of the adsorbing surface of the

precipitated globulins, but there seems to be some difficulty in understanding why the albumins of the fresh serum do not effect a solution of the globulins, thus setting the adsorbed complement free, such as occurs on the addition of a normal E-piece. It may be possible also to assume, that addition to a fresh serum of such a dispersed phase as an opalescent shaken serum represents gives rise in the fresh serum to adsorptions by increase of surface analogous to those which effected the inactivation by shaking.

The reactivation of a shaken serum by means of the complement fractions.

As regards the technique it must be mentioned, that in order to obtain the complement fractions, the CO_2-method of Liefmann has been employed, it giving more uniform results than the HCl-method of Altmann, which I had previously employed. After diluting the serum 1 : 8 with aqu. dist., CO_2 was passed through for half an hour, the temperature being kept low by means of ice. The serum was then well centrifuged, the supernatant fluid filtered through hardened paper and then rendered isotonic. The precipitate was washed once with distilled water and diluted with 0·85 % NaCl solution immediately before use.

For the sake of brevity I shall employ the following signs :

C_n = normal complement containing serum.
C_{sh} = serum inactivated by shaking at 37° C.
C_{th} = serum inactivated by heating at 56° C. for half an hour.
C_5, C_{10}, etc. = sera inactivated by heating at 56° C. for 5, 10, etc. minutes.
E_n and M_n = CO_2 fractions obtained from a normal serum (C_n).
E_{sh} and M_{sh} = CO_2 fractions obtained from a shaken inactivated serum (C_{sh}).
E_{th} and M_{th} = CO_2 fractions obtained from a thermo-inactivated serum (C_{th}).
E_5, E_{10}, etc. and M_5, M_{10}, etc. = CO_2 fractions from sera heated at 56° C. for 5, 10, etc. minutes.

Ritz (1912) showed that the process of inactivation by shaking consists of two stages. In the first stage it is possible to reactivate C_{sh} by M_n or E_n, in the second stage this has become impossible. I found previously the reactivation by M_n to be impossible, and if we are to understand by reactivation a complete restitution of an

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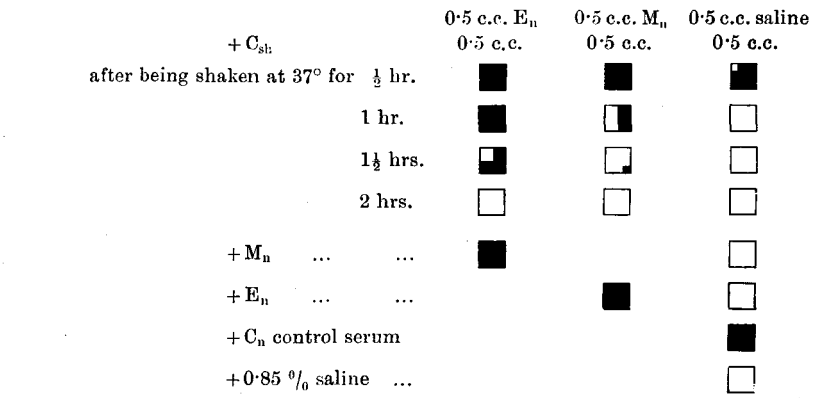
otherwise completely inactivated shaken serum, I must still adhere to that opinion; but that a partial reactivation can be effected by M_n is to be seen from the experiments I and II.

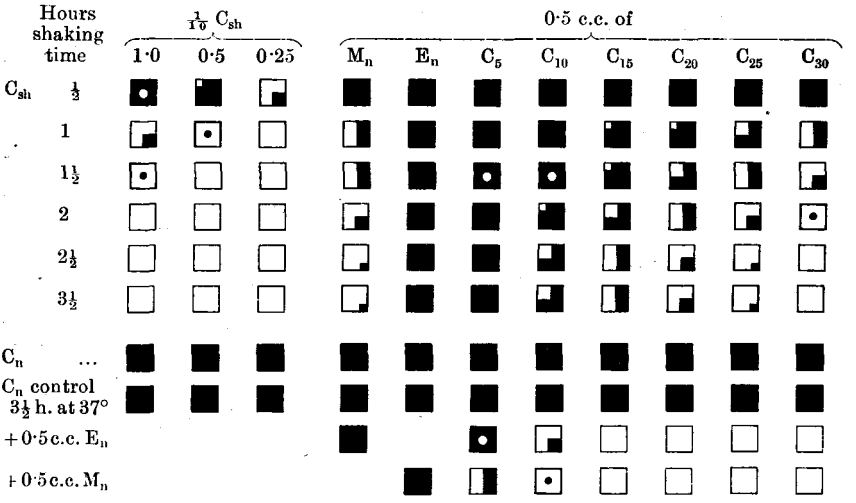
Exp. I. Guinea-pig serum, diluted 1 : 10 with 0·85 % saline, was shaken for different times, then tested as regards its haemolytic power and its reactivation by M-piece and E-piece.

10 c.c. of this serum was shaken in tube of 60 c.c. volume.

Haemolysis of 0·5 c.c. sensit. red cells (AB dose = three times the single lysing dose) after 1 hour at 37° and 15 hours at room temperature.



Exp. II. C_n diluted $\frac{1}{10}$ with saline was shaken; 13 c.c. liquid in 67 c.c. containing tube.



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Every half an hour 1·5 c.c. was taken out, centrifuged and tested.

C_n was undiluted, exposed to 56° C. for between 5 and 30 minutes, then diluted 1 : 10 with saline.

(AB dose and amount of red cell emulsion equal to Exp. I.)

Haemolysis after 1 hour at 37° and 15 hours at room temperature.

To 0·5 c.c. of C_{sh} was added 0·5 c.c. of M_n, E_n or C_n, the total volume being 1·5 c.c.

These experiments I and II, as well as many others giving similar results, demonstrate that a serum, the complementing power of which is considerably diminished by the shaking, but which is yet not completely inactivated, can be fully restored by M_n. If however the inactivation process is further advanced and finally completed, the haemolytic action of the serum can no longer be restored by M_n, except to a very small degree, while E_n has still its full reactivating effect. Further shaking of the serum will diminish and finally abolish the possibility of reactivation by E_n, and, as Exp. II shows, also that of C_{th}. Therefore the inactivation process by shaking is to be considered as progressing in such a manner that the possibility of reactivation by M_n is first lost, later that of E_n, and finally the reactivation by C_{th} disappears. Such a serum is then irreversibly inactivated.

The reactivation of a serum inactivated by shaking, by means of a thermo-inactivated serum and the relation of this process to the reactivation by the normal complement fractions.

The reactivation of C_{sh} by C_{th} is in some way connected with the reactivation by means of M_n and E_n, but before attempting to explain this, I will give the results of a series of experiments demonstrating these facts.

The above-mentioned Exp. II shows that the possibility of reactivating C_{sh} by C_{th} decreases progressively, either if the C_{th} is heated for a longer and longer time, or if the C_{sh} is submitted to a more prolonged shaking.

In a former paper (1913) I pointed out that, generally speaking, C_{sh} can be reactivated by C_{th} as long as the latter can be reactivated by E_n. I shall refer to this later. In Exp. II however this is only approximately the case, for it is shown that C_{15} is able to reactivate a C_{sh} shaken 2 hours, to a large extent, in spite of the fact that neither E_n nor M_n has any effect on C_{th}. This is exactly what Ritz explains by assuming a third component.

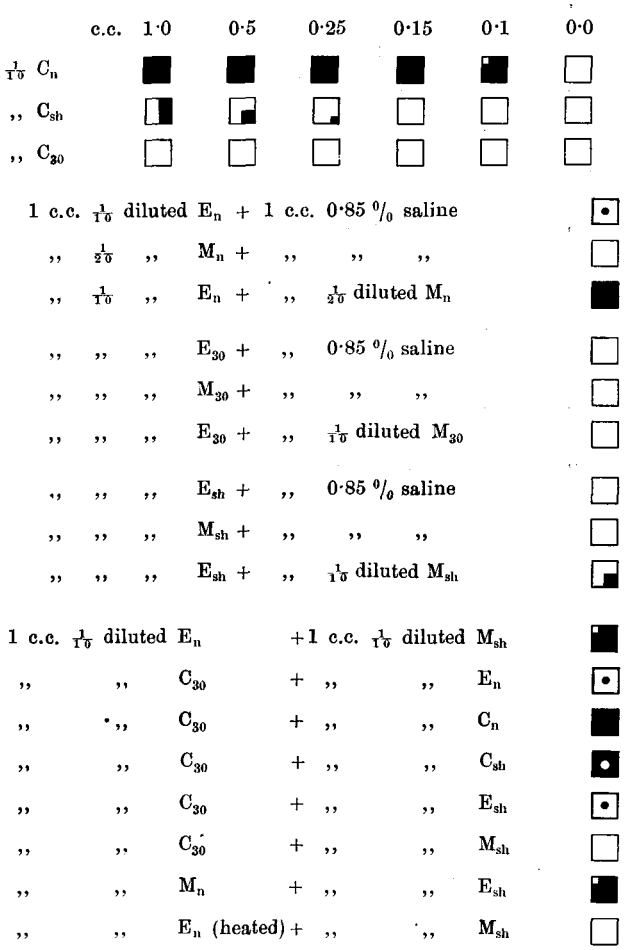
Exp. III. A guinea-pig serum of 24 hours standing was partly shaken at 37° undiluted, partly heated at 56° for half an hour and partly not treated.

All sera were then submitted to the effect of CO_2 and thus the fractions were obtained, the signs for which are employed as above stated.

Haemolysis of 1 c.c. sensit. red cell emulsion after 1 hour at 37° and 15 hours at room temperature.

(AB dose = four times the single lysing dose.)

Exp. III shows that C_{sh} can be reactivated by M_n, the more so the greater the dilution of M_n, and that M_{30}, *i.e.* a M-piece derived



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1 c.c.	$\frac{1}{10}$	diluted	C_{sh} + 1 c.c.	$\frac{1}{2}$	diluted	M_n	...	
,,	,,	,, +	,,	$\frac{1}{4}$	,,	,,	...	
,,	,,	,, +	,,	$\frac{1}{20}$	,,	,,	...	
,,	,,	,, +	,,	$\frac{1}{2}$	,,	M_{sh}	...	
,,	,,	,, +	,,	$\frac{1}{4}$	,,	,,	...	
,,	,,	,, +	,,	$\frac{1}{10}$	,,	,,	...	
,,	,,	,, +	,,	$\frac{1}{2}$	,,	M_{30}	...	
,,	,,	,, +	,,	$\frac{1}{4}$	,,	,,	...	
,,	,,	,, +	,,	$\frac{1}{10}$	,,	,,	...	
,,	,,	,, +	,,	$\frac{1}{10}$	,,	E_n	...	
,,	,,	,, +	,,	$\frac{1}{20}$	,,	,,	...	
,,	,,	,, +	,,	$\frac{1}{40}$	,,	,,	...	
,,	,,	,, +	,,	$\frac{1}{10}$	,,	E_{sh}	...	
,,	,,	,, +	,,	$\frac{1}{20}$	,,	,,	...	
,,	,,	,, +	,,	$\frac{1}{40}$	,,	,,	...	
,,	,,	,, +	,,	$\frac{1}{10}$	,,	E_{30}	...	
,,	,,	,, +	,,	$\frac{1}{20}$	,,	,,	...	
,,	,,	,, +	,,	$\frac{1}{40}$	,,	,,	...	
,,	,,	,, +	,,	$\frac{1}{10}$	,,	E_n (heated)		

from a thermo-inactivated serum, gives a more complete reaction than M_n, M_{sh} having no effect.

In the case of the reactivation by E-piece it is irrelevant whether the E-piece is derived from a normal or from a thermo-inactivated serum. Even the addition of E_{sh} has a marked effect.

The thermo-inactivated serum, C_{30}, was not anti-complementary nor could it be reactivated by E_n or by E_{sh}, except to a very slight extent. On the other hand its reactivating power on C_{sh} was well marked, the latter being however not completely inactive.

Exp. IV. Fresh guinea-pig serum was treated with CO_2, and the M-piece thus obtained shaken for 1 hour at 37°; other parts of the M-piece were diluted in different concentrations and then either heated or not treated otherwise.

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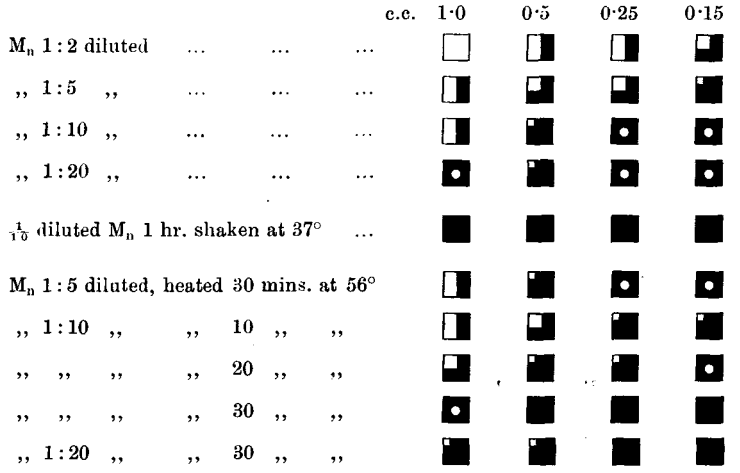
Each tube contains 1 c.c. $\frac{1}{10}$ diluted C_n, M-piece in decreasing amounts, saline to fill up to 2 c.c. and 1 c.c. sensit. red cell emulsion.

(AB dose = three times the single lysing dose.)

Haemolysis after 1 hour at 37° and 24 hours standing at room temp.

The control reactions were satisfactory.



Exp. IV shows the anti-complementary effect of a normal M-piece on a normal serum, decreasing with dilution and with heating, the shaken M-piece having no longer any anti-complementary effect.

Exp. V. Haemolysis after 1 hour at 37° and 15 hours standing at room temp.

1 c.c. sensit. red cell emulsion, AB dose being three times the single lysing dose.

Total volume in each tube = 3 c.c., of each factor 1 c.c. being in reaction.

From Exp. V it follows that a shaken serum which is completely inactivated and unable to be reactivated by a normal M-piece is restored by the addition of a M-piece obtained from a thermo-inactivated serum.

The collected froth of a shaken serum retains its haemolytic power longer than the shaken liquid.

No difference is to be found in the reactivation of a shaken serum by an E-piece, whether obtained from a normal serum or from a thermo-inactivated serum.

In the course of thermo-inactivation the property of being reactivated

1 : 10 diluted	E_n	+	saline ...	
,,	M_n	+	,, ...	
,,	E_n	+	M_n $\frac{1}{10}$ diluted	
,,	E_5	+	saline ...	
,,	M_5	+	,, ...	
,,	E_5	+	M_5 $\frac{1}{10}$ diluted	
,,	E_{30}	+	saline ...	
,,	M_{30}	+	,, ...	
,,	E_{30}	+	M_{30} $\frac{1}{10}$ diluted	
,,	M_n	+	E_5 ,, ,,	
,,	,,	+	E_{30} ,, ,,	
,,	E_n	+	M_5 ,, ,,	
,,	,,	+	M_{30} ,, ,,	
,,	C_n	+	M_n $\frac{1}{2}$,,	
,,	,,	+	M_5 ,, ,,	
,,	,,	+	M_{30} ,, ,,	
,,	C_{sh}	+	M_n ,, ,,	
,,	,,	+	,, $\frac{1}{10}$,,	
,,	,,	+	M_5 $\frac{1}{2}$,,	
,,	,,	+	,, $\frac{1}{10}$,,	
,,	,,	+	M_{30} $\frac{1}{2}$,,	
,,	,,	+	,, $\frac{1}{10}$,,	
,,	,,	+	saline ...	
,,	collected froth of C_{sh}	+	,, ...	
,,	C_{sh}	+	E_n $\frac{1}{10}$ diluted	
,,	,,	+	E_5 ,, ,,	
,,	,,	+	E_{30} ,, ,,	

by the normal complement fractions is lost quicker in the case of the M-piece than in the case of the E-piece.

The anti-complementary effect produced on normal complement by

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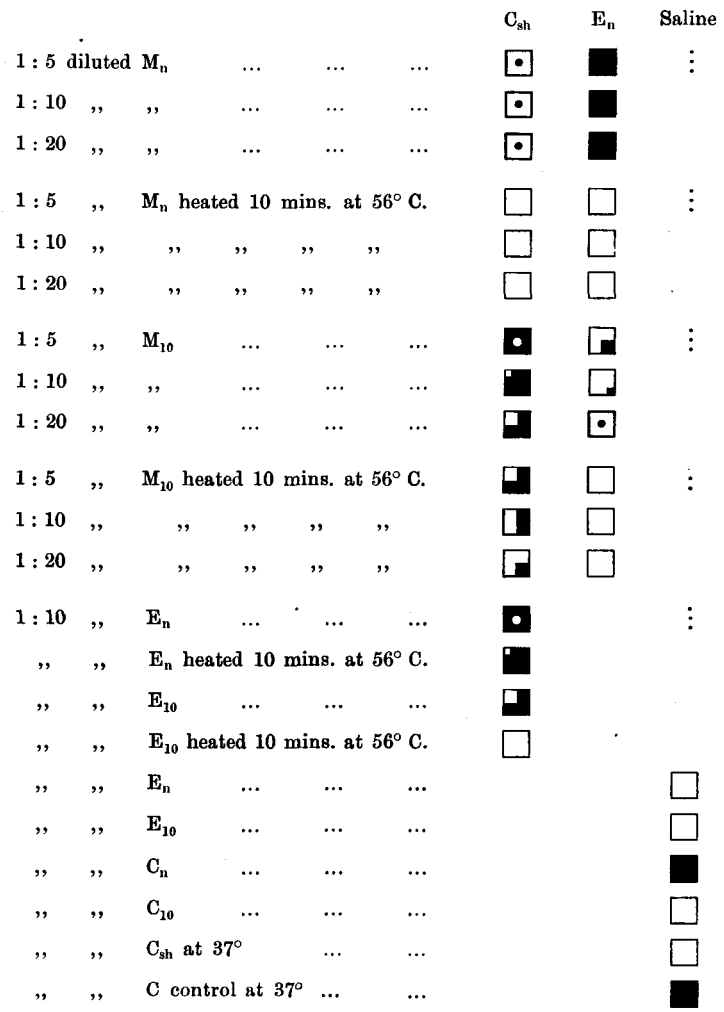
a M-piece obtained from a thermo-inactivated serum is very small when compared with the inhibiting effect of a normal M-piece.

Exp. VI. Haemolysis after 1 hour at 37° and 15 hours at room temperature.

1 c.c. sensit. red cell emulsion. AB dose = three times the single lysing dose.

Total volume in each tube = 3 c.c., of each factor 1 c.c. being present.



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This experiment shows first of all the great difference in the action of a M-piece upon an E-piece or on a shaken inactivated serum. Exp. VI shows also what we have already seen in Exp. V, that a shaken inactivated serum, not being reactivated by a normal M-piece, will become so, if the M-piece is derived from a thermo-inactivated serum, exposed to 56° C. for 10 minutes. Such a M-piece has a slight effect, when combined with a normal E-piece, but while heating such a M-piece will completely destroy this effect, the action of it on a shaken serum is but slightly diminished.

The same experiment shows further, that the effect of heating an isolated E-piece will not altogether destroy its power of reactivating a shaken serum, but it will do so, if the E-piece has been obtained from a serum already heated for 10 minutes at 56° C.

It is of interest to note in this connection, that Exp. III shows that a M-piece obtained from a shaken but not completely inactive serum acted quite well with a normal E-piece, but did no longer do so if the latter had been heated.

Exp. VII. Haemolysis of 1 c.c. sensit. red cell emulsion (AB dose = three times the single lysing dose) after $1\frac{1}{2}$ hours at 37°.

$\frac{1}{10}$ diluted E_n, c.c.	1·0	1·0	1·0	1·0	0·0
0·85 % saline, c.c.		0·5	0·75	0·85	1·0
+ c.c.	1·0	0·5	0·25	0·15	1·0
$\frac{1}{10}$ diluted M_n (of C_n)	■	■	■	◉	□
,, ,, M_n shaken at 37° ...	□	□	□	□	□
,, ,, M_n control at 37° ...	◪	◪	⊡	□	□
,, ,, M_{sh} (of C_{sh})	■	■	◉	◪	□
,, ,, M_n (of C control) ...	■	■	■	◉	□
$\frac{1}{10}$ diluted M_n, c.c.	1·0	1·0	1·0	1·0	0·0
0·85 % saline, c.c.		0·5	0·75	0·85	1·0
+ c.c.	1·0	0·5	0·25	0·15	1·0
$\frac{1}{10}$ diluted E_n	■	■	◪	◪	□
,, ,, E_n shaken (isotonic) 37°	■	■	◪	⊡	□
,, ,, E_n ,, (not isotonic) 37°	■	■	◪	◪	□
,, ,, E_n control (isotonic) 37°	■	■	◉	◪	□
,, ,, E_n ,, (not isotonic) 37°	■	■	◉	◪	□
,, ,, E_{sh} (of C_{sh})	■	◉	◉	□	□
,, ,, E_n (of C control) ...	■	■	■	◪	□

	0·85 % saline, c.c.		0·5	0·75	0·85	0·9
	+ c.c.	1·0	0·5	0·25	0·15	0·1
$\frac{1}{10}$ diluted C_n						
,, ,, C_{sh} at 37°						
,, ,, C control at 37° ...						

From the observations made in Exp. VII the following conclusions can be drawn :

The normal action of a M-piece on an E-piece is abolished by shaking the isolated M-piece. If the M-piece however is derived from a shaken serum, the complementing power of which is considerably weakened by shaking, such a M-piece still has a well-marked effect with a normal E-piece.

On the other hand, if the action of a normal E-piece on a normal M-piece is considered, no loss of activity of the E-piece is observed when the latter has been shaken for an equal length of time as the M-piece.

[Only shaking the E-piece for a very long time will destroy its action.] The E-piece of a shaken serum is altered only to a very slight extent.

Exp. VIII. Fresh guinea-pig serum $\frac{1}{10}$ diluted was shaken 4 hours at 37°, then centrifuged and submitted to the following tests :

Haemolysis of 0·5 c.c. sensit. red cell emulsion (AB dose = twice the single lysing dose) after 1 hour at 37° and about 15 hours at room temperature.

E_n B = E-piece, which after being rendered isotonic, was filtered through a Berkefeld filter (40 × 14 mm.).

E_n A = E-piece, the reaction of which has been rendered equal to that of the C_n by means of addition of $\frac{n}{250}$ NaOH, neutral red and rosolic acid being used as indicators.

From Exp. VIII it follows that the reactivation of a shaken inactive serum by means of an E-piece is only slightly diminished if the latter has been filtered through a Berkefeld filter. [This observation is not constantly found, cf. Exp. IX.] But the action of the E-piece on a M-piece is totally abolished by the Berkefeld filtration.

A normal E-piece reactivates a shaken serum even after being heated 15 minutes, but a slight addition of alkali to the E-piece destroys its action, whether heated or unheated, on a shaken serum as well as on a normal M-piece [not constantly found].

1 c.c. $\frac{1}{10}$ diluted C_{sh} + 2 c.c. $\frac{1}{10}$ diluted E_n	■
,, ,, ,, + 1 ,, ,, E_n	■
,, ,, ,, + 2 ,, ,, E_nB	■
,, ,, ,, + 1 ,, ,, E_nB	■
,, ,, ,, + 2 c.c. $\frac{1}{20}$,, M_n	□
,, ,, ,, + 1 ,, ,, M_n	□
,, ,, ,, + 2 c.c. $\frac{1}{10}$,, E_n heated for 15 mins. at 56° C.	■
,, ,, ,, + 2 ,, ,, E_nA ,, ,, ,,	□
,, ,, ,, + 2 ,, ,, C_n ,, ,, ,,	■
,, ,, ,, + 2 ,, ,, E_nA	□
,, ,, ,, + 1 ,, ,, E_nA	□
1 c.c. $\frac{1}{20}$ diluted M_n + 1 c.c. $\frac{1}{10}$ diluted E_n	■
,, ,, ,, + 1 ,, ,, E_nB	□
,, ,, ,, + 1 ,, ,, E_nA	□
,, ,, ,, + 1 ,, ,, E_n heated for 15 mins. at 56° C.	□
,, ,, ,, + 1 ,, ,, E_nA ,, ,, ,,	□
1 c.c. $\frac{1}{10}$ diluted C_{sh} + 1 c.c. saline	□
,, ,, C_n + ,, ...	■
,, ,, C_{th} (15 mins. at 56°) + ,, ...	□

Haemolysis of 0·5 c.c. sensit. red cell emulsion after 1 hour at 37° and 15 hours at room temperature; sensitization effected with three times the single lysing dose of AB.

Exp. IX shows that a shaken serum can no longer be reactivated by an E-piece, if submitted to Berkefeld filtration. Contrarily to Exp. VIII this experiment shows that the E-piece after Berkefeld filtration was no longer capable of reactivating a shaken serum.

If an E-piece is heated for too long a time its action on a shaken serum will disappear.

Addition of alkali to an E-piece has a somewhat lesser inhibiting effect in this experiment than in Exp. VIII.

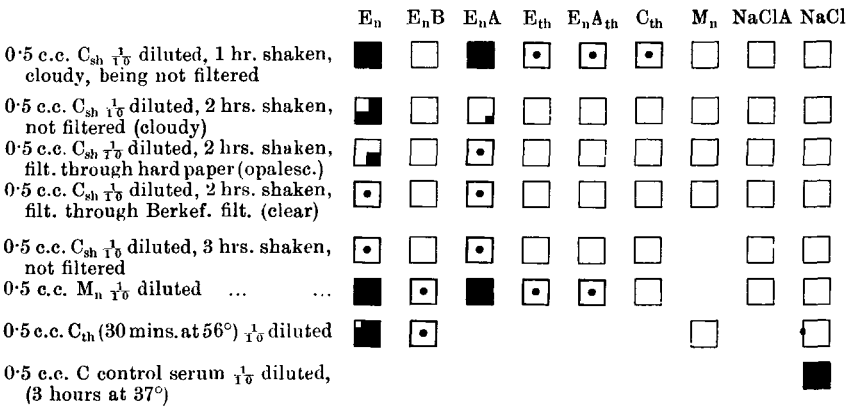
A thermo-inactivated serum, in spite of being reactivated by an E-piece, showed no action on the shaken inactive serum.

Experiment IX.

E_nA = 0·5 c.c. E_n + 0·3 c.c. $\frac{n}{250}$ NaOH in 0·85 % NaCl in each tube.

E_nA_{th} = 0·5 c.c. E_n + 0·3 c.c. $\frac{n}{250}$ NaOH in 0·85 % NaCl, the mixture heated for 30 mins. at 56° C.

NaCl = 0·5 c.c. NaCl solution + 0·3 c.c. $\frac{n}{250}$ NaOH in 0·85 % NaCl in each tube.



Summary of the experimental results.

In order to better survey the results obtained in the experiments described, I summarise them as follows :

A shaken serum can be reactivated by M_n, E_n, C_{th} or not at all, according to the degree of inactivation produced by the shaking [I, II].

The restitution effect of M_n is only found if the activity of the serum has not yet been completely destroyed [I, II]. This action of M_n disappears when the isolated M_n has been shaken or heated [VI], but a M-piece of a C_{th} may still exert full reactivation on a C_{sh}, when M_n no longer produces such an effect [V, VI]. Even heating a M_{th} will influence but little its restitution effect on a C_{sh} [VI]. Normally M_n has an anti-complementary effect upon C_n [IV]. This effect will be diminished by increasing dilution of the M_n and with the influence of heat upon it, but it will altogether disappear if M_n has been shaken [IV]. The normal action of M_n upon E_n will also be destroyed either by shaking or heating of the M_n [VII]. A M_{th} however still exerts some action upon E_n, which promptly disappears, if such an isolated M_{th} is exposed to heat. But even then it retains some of its reactivating power on a C_{sh} [VI].

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A shaken serum completely inactivated as regards complement action can still be reactivated by E_n or C_{th}, when M_n will no longer have any effect [I, II]. It requires a much longer time to render an E_n inactive by shaking [VII] than it does for a M_n. An E_n works equally well as regards reactivation of C_{sh}, whether obtained from a C_n or C_{th} [V, VI]. If an isolated E_n be heated, its power of reactivating a shaken inactive serum is maintained for a longer time than its activity with a M_n [VIII]. The addition of alkali to an E_n, which obtained by CO_2 is acid in character, tends to inhibit its restitution effect on C_{sh}, but this result has so far not been obtained with regularity.

The reactivation of C_{sh} by means of C_{th} can take place, in spite of C_{th} not being able to be reactivated by M_n or E_n. The property of C_{th} to reactivate a C_{sh} decreases with a prolonged exposure to heat. *Vice versa,* however, a C_{th} may be reactivated by an E_n and yet be without effect on C_{sh} [IX]. In such a case no anti-complementary effect of C_{th} on a normal C_n could be observed [II].

By means of Berkefeld filtration it is possible to render a C_{sh} no longer able of being reactivated by E_n [IX], and on the other hand to allow an E_n still to retain its action on C_{sh} [VIII].

Theoretical part.

First of all I think it certain that shaking produces a progressing alteration of all the serum proteins, leading finally to their denaturation. This alteration affects principally and first of all the euglobulin, changes in the albumin and pseudoglobulin following later, but always being present before visible coagulation takes place. There is not yet enough evidence to identify the complementing property of a serum with a certain definite state of equilibrium in the physical conditions of the different proteins, as regards their quantity and their degree of dispersity, which latter are closely connected with the amount of salts present and the $H^{\cdot}$-concentration. But it can be safely assumed that complement action, whether due to an existing substrate or not, is dependent on certain physical conditions of the serum and will therefore become lost as a consequence of anything which alters these conditions. The reactivation of a serum is only a question of how far these alterations are reversible or not.

Now, the following explanation may cover at least the majority of observations, if not all.

Addition of a M-piece to a shaken serum can only produce reactivation if the alteration, which the albumins undergo by shaking, is not yet so far advanced as to prevent them from controlling the dispersity of euglobulin. It is evident that irreversible changes in the added M-piece brought about by shaking or heating destroy any effect which the addition of a M-piece may otherwise have. Alterations in the albumins in a serum produced by shaking take place more rapidly than the denaturation of the euglobulin, which latter process is only the consequence of the former, as I explained in greater detail in another communication [1914].

From this it follows that reactivation by M-piece very soon disappears, and that addition of an E-piece has an effect only so long as there are euglobulins still in a reversible condition of commencing denaturation. Their removal by a Berkefeld filtration abolishes any reactivation by an E-piece, but the Berkefeld filtration of an E-piece is not necessarily followed by a loss of its restitution power, for I found that Berkefeld filtration chiefly retains the pseudoglobulin of the E-piece (1914). Such a Berkefeld filtered E-piece shows no longer any activity with a normal M-piece, which is difficult to explain. This may be due to the absence of pseudoglobulins, which according to the recent work of Browning and Mackie (1914) represent the main factor in the haemolytic complementing action of a serum. I could however not obtain these results with well dialysed preparations of the different proteins of guinea-pig serum, so I must leave this question undecided. On the other hand, I found that addition of pure albumin obtained by fractionated salting out with the sulphate of ammonium has a similar but weaker effect on a shaken inactive serum than that of an E-piece [not constantly found]. To destroy this power of E-piece by shaking it requires a longer time, owing to its solubility in water being greater than that of the euglobulin.

If the shaking effect however is so advanced that no more euglobulin is left in a reversible state, the addition of both, E-piece as well as pure albumin, will have no more effect. If this be the case, sometimes a thermo-inactivated serum is found to have still a marked effect on the reactivation of a shaken serum, but before attempting to explain this fact the alterations of a fresh serum produced by heat must be considered.

As I described in greater detail in another communication, heating a serum to 56° C. for half an hour is followed by more or less marked denaturation of its proteins [H. Chick and C. J. Martin, 1913]. The $H^{\cdot}$-concentration is decreased and the heat denaturated particles are

kept in dispersion by their negative charge. If by acidification with CO_2 the particles become isoelectric with the medium, they aggregate and form a precipitate. The precipitate thus obtained (M_{th}) contains not only euglobulin with some pseudoglobulin, which form the precipitate by CO_2 in the case of a normal serum diluted with water, but it contains that portion of all proteins which has been affected by the heat. In redissolving the precipitate in saline, a part of it is often found to be insoluble, probably consisting chiefly of euglobulin. Such a M_{th} due to its albumin content can still reactivate a shaken serum upon which a normal M-piece will no longer have any effect. Perhaps it is the electrical charge of its particles and also its albumin content which renders such a M_{th} more resistant to heat than a normal M-piece.

If a serum is exposed to 56° C., its complementing power rapidly decreases with the length of time, and this happens a little sooner in the case of an undiluted serum, due probably to the ratio of the protein content to the salt concentration being higher in the case of a diluted serum, and to the fact that the presence of salts lower the coagulation rate by heat [Chick and Martin].

If the time of exposure to 56° C. be varied, and the different samples of sera so obtained be treated by CO_2 in order to obtain the M-piece or E-piece, it is found by their combination with the corresponding fractions of a normal serum [cf. Exp. V] that first the E-piece, later the M-piece, is destroyed, *i.e.* that an action of a M_{th} in combination with an E_n can be obtained for a greater length of time than the action of an E_{th} with a M_n. This has been already found by many other authors [Sachs, 1913]. From this it follows that as long as a shaken inactivated serum can be reactivated by a normal E-piece, it can also be reactivated by a thermo-inactivated serum, if the latter is capable of being reactivated by an E-piece. In some cases however such a thermo-inactivated serum is found to have no influence on a shaken inactivated serum, in spite of both being reactivated by a normal E-piece. I think that alterations in the $H^{\cdot}$-concentration account for this phenomenon.

On the other hand, a thermo-inactive serum, incapable of being restored by an E-piece, may still reactivate a shaken serum. I assume that such a thermo-inactivated serum may have the faculty of bringing even the denaturated euglobulins back into solution, perhaps owing to its alkalinity. A similar faculty of lysing a precipitate is attributed to a heated serum by Welsh and Chapman (1909) in explaining their observation of the inhibition which a heated serum exerts on specific precipitation. If however the denaturation of the shaken proteins has

advanced too far, even this lysing effect of a heated serum can no longer be obtained.

There are other observations more difficult to explain, for instance the fact that a M-piece of a serum, heated for 10 minutes at 56° C., gives a better reaction with a shaken serum than with a normal E-piece [Exp. VI], and further, that 10 minutes heating of such a M_{th} will abolish the effect on an E-piece but only slightly diminish the action with a shaken serum. I think it very probable that here again alterations in the H·-concentration play the most important part. Some preliminary investigations have shown that addition of traces of acid or alkali do not inhibit the effect of shaking, and on the other hand I found it impossible to reactivate a shaken serum by means of decreasing or increasing its H·-concentration. I intend however to continue my research on these lines, since I can confirm Noguchi's and Bronfenbrenner's observation, that it is possible only by means of adding some alkali to an E-piece, obtained by Liefmann's method, to restore full haemolytic action. But I shall deal with this in a later communication.

As already mentioned, P. Schmidt considers complement to be a ferment, an entity, which is adsorbed on the globulin surfaces in all processes, leading to their precipitation. I readily recognise how easily the main features of the processes involved in the shaking inactivation can be explained by this conception, but there are many points, the explanation of which by this hypothesis seems to be very difficult if not impossible. The following are some of these points :

P. Schmidt's conception does not explain the reason why the albumins in the shaken serum fail to bring the denaturated particles into solution, whereas fresh albumin will do so. Further the reactivation of C_{sh} by C_{th}, the fact that M_{th} has a reactivation effect on C_{sh}, whereas M_n is ineffective, and that such a M_{th} after being heated loses its activity with E_n but not with C_{sh}. The observation that an E_n being submitted to Berkefeld filtration remains active with a C_{sh}, but not longer with a M_n.

Last but not least the observation that a serum, submitted to the effect of dilution and standing, becomes inactive, if rendered isotonic, in spite of the euglobulins being in complete solution, etc.

I claim on the other hand to have shown, that at least as many of the observations can be equally well explained by assuming no substrate for a complement at all, but the necessity of certain physical conditions. I hope later to be able to bring forward more evidence in support of this conception.

General conclusions.

It has been attempted to show that at least most of the experimental results, a summary of which is already given above, can be explained by assuming that shaking produces alterations of the physical conditions in the serum, which by themselves account for the loss of complementing power of the serum, and that the conception of a complement being a ferment, the action of which is due to the presence of a certain substrate, is not necessary. But there are some observations left unexplained by either conception, due to lack of further experimental evidence.

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