THE INACTIVATION OF COMPLEMENT BY MECHANICAL AGITATION.

By HANS SCHMIDT.

(*From the Bacteriological Department, Lister Institute, London.*)

CONTENTS.

INTRODUCTORY.

OF the many methods employed to render a serum inactive, that by means of mechanical agitation has been the subject of several recent works, but the real nature of this phenomenon, as it occurs in shaken sera, is still unknown. The reason is the uncertainty regarding the nature of complement itself, and even the most satisfying explanation of the inactivation of complement by shaking would not probably throw much light on the nature of the complement. I give in the following

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a brief summary of the literature dealing with the inactivation of complement by mechanical agitation.

The first systematic experiments to render a serum inactive by shaking were undertaken by Jakoby and Schuetze (1909). These authors found (1910) that mechanical agitation renders complement inactive, the time necessary for the effect being shorter the higher the temperature at which the shaking takes place. By this procedure the serum becomes dim and a precipitate occurs, but sera which were not completely inactivated, also showed dimmess, so that the authors concluded that the inactivity cannot be due solely to the coagulation and precipitation of proteins. A serum does not lose the property of being inactivated by shaking if it is frozen and freezing does not reactivate a shaken inactive serum. The latter can however be reactivated by both fractions of the complement either by end-piece or by mid-piece, and if the serum is centrifugalised the precipitate can be reactivated by both fractions, while end-piece only is capable of reactivating the supernatant fluid. Shaken inactive serum does not inhibit normal complement action. The experiments of these authors were in part confirmed by Zeisler (1909), Stuehmer (1910), Noguchi and Bronfenbrenner (1911). These papers however did not add new facts to those already known by Jakoby and Schuetze, but an elaborate paper of Ritz (1912) gave to many points a better explanation. Ritz, with a better shaking apparatus, succeeded in shortening considerably the time necessary for inactivation, so that 20 to 30 minutes shaking was sufficient, while Jakoby and Schuetze were obliged to shake six hours and longer to render a serum inactive. He confirmed also the accelerating action of the temperature. Jakoby and Schuetze took into consideration the difference between ordinary and Jena glass as affording some explanation of the fact, that some sera could be inactivated in a relatively short time while others were very resistant to the shaking effect, and they believed that the discrepancy might be due to the alkali produced from the ordinary glass. Ritz (1912) however accentuated the important *rôle*, which the relation between the volume of the tube and the quantity of the shaken liquid plays in the process. He proved experimentally that even small differences (100–110 c.c.) between the volumes of different tubes—the quantity of shaken liquid being the same—influence very greatly the time necessary for inactivation by shaking, thus believing that the variant results in the experiments of Jakoby and Schuetze are due to these circumstances. Ritz found by examining the relation between the volume of the tube and the quantity and concentration of the liquid,

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that with a concentration 1:10 of serum the rapidity, with which inactivation by shaking is achieved, is optimal. With regard to the reactivation of shaken inactive serum by the complement fractions, Ritz was able to confirm the results of Jakoby and Schuetze, but he showed that this reactivation depends upon the time during which the serum is exposed to agitation. Long periods of shaking render the serum incapable of being reactivated. He further showed, contrary to Jakoby and Schuetze, that the precipitate cannot by any means be reactivated. In some experiments Ritz succeeded in reactivating shaken inactive serum by adding thermoinactive serum, but this effect of the latter is reduced to a minimum if the shaking is continued for a long time. This phenomenon has been confirmed by Kashiwabara (1913), but not in all sera. The time he used for thermoinactivation was always half an hour at a temperature of between 50·7° and 60°. This exposure to heat is probably too long and deprives the serum of its property to reactivate shaken serum. In further experiments Kashiwabara showed, that the reactivation of shaken serum by mid- or end-piece is no longer possible if these fractions have been separately shaken before use. In a mixture of one fraction shaken with the other unshaken, complement action however can be restored, if the latter fraction is sufficiently concentrated. The author found, that thermoinactive serum, if shaken before or after the treatment by heat, loses its property of reactivating shaken-inactive serum. Finally I would mention the experiments of Courmont and Dufour (1912). These authors (1912) showed, that sera inactivated by shaking do not become anticomplementary like sera inactivated by heat. Experiments undertaken with the object of studying the influence of the gas with which the serum is shaken led the authors to the conclusion, that the oxygen plays an important part in the inactivation.

It is chiefly with the object of ascertaining whether confirmation could be obtained of this important statement of Courmont and Dufour that the following investigations were undertaken.

Technique of experiments.

Not having at my disposal an Uhlenhuth's kinotherm such as Ritz used for his experiments, I employed an ordinary shaking apparatus, the electro-motor of which was provided with a resistance to regulate the velocity. Further the apparatus was fitted with an arrangement for altering the distance, along which the shaken tube was moved. In the following experiments this distance was always 7·8 cm. The serum was

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shaken in a dark room at a temperature of between 33°–36° C. I used cylinder-shaped tubes closed by rubber stoppers. Many controls showed me, that the contact of the serum with the carefully cleaned rubber did not alter the properties of the serum. Only the haemolytic complement of guinea-pig serum has been examined. Its complementary function was tested by its haemolytic power in combination with sensitized, three times washed, sheep red corpuscles of which a 5 % emulsion in 0·85 % saline solution was used. The amboceptor was inactivated rabbit serum, the single lysing dose of which was 0·00125 c.c. by using 1 c.c. of the red cell emulsion and 0·1 c.c. complement. In the haemolytic tests the following scheme is used to illustrate different degrees of haemolysis.

No haemolysis ... ...
Trace haemolysis... ...
Slight „ ... ...
Half haemolysed ... ...
Strong haemolysis ...
Almost complete haemolysis
Complete haemolysis ...

To split the complement-containing serum into the so-called mid- and end-piece I used the procedure of Sachs and Altmann (1908) in the following way:

1·0 c.c. undiluted complement serum plus 8·2 c.c. n/300 HCl in aqu. dist. was kept for 1 hour at room temperature, then centrifugalised and the sediment instantly—to avoid Brand's modification—washed three times with distilled water. 10 c.c. of 0·85 % saline solution were then added. The supernatant fluid, after being filtered, was neutralised and rendered isotonic by adding 0·8 c.c. of an n/30 (NaOH) solution which contains 10 % saline. The result is that both fractions are in a dilution of 1 : 10 and that the mixture of 1·0 c.c. of each corresponds to 0·1 c.c. of each original complement-containing serum. I obtained good results by this procedure in most cases. Sometimes the supernatant fluid (end-piece) still retained some lytic property, but it could be avoided by allowing the serum to remain 15 minutes to half an hour longer in contact with the hydrochloric acid.

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Influence of time, temperature and proportion between volume of tube and volume of fluid.

The dependence of the shaking inactivation upon time and temperature could be confirmed as follows: at low temperatures, 0–20°, shaking must be continued a very long time before the complement action is perceptibly weakened. Occasionally fresh serum is practically not affected by shaking at low temperature. The time necessary for inactivation by shaking decreases rapidly with rise of temperature. In my experiments however the average time required to render the serum completely inactive was 4–6 hours at 33° C. Other factors however, such as the concentration of the serum and the relation between the volume of the tube and the quantity of shaken liquid, influence greatly the time necessary for inactivation. Ritz (1912) had already laid much stress on these factors. I too attempted to determine experimentally the optimum of the concentration and of the relation between the volumes of tube and liquid in regard to the shaking inactivation, but I met with considerable difficulties, as I shall explain in the following:

The tubes I used for shaking were cylinders of different width and length. Now if the temperature, the velocity, and the distance of the shaking movement, as well as the diameter and the length of the tube are kept constant, there are still two variables left, viz. the concentration of the serum and its quantity, and, as a function of both, the absolute amount of complement. The latter is naturally altered, if the quantity of the serum is kept constant and the concentration varies, but if the concentration of the serum is kept constant and the quantity changed, the absolute amount of complement is still different in each tube. If instead of a serum, containing complement in a certain concentration, only pure water is considered, the problem is reduced, so that it can be more easily treated from a purely physical standpoint. Thus the problem would be the following:

In a given cylindrical vessel, water is to be shaken with air under constant conditions. What must be the relation between the quantity of water and the volume of the tube to render the intensity of shaking a maximum?

This problem is not soluble experimentally because no criterion exists to judge the degree of shaking intensity. But also theoretically I was unable to find a solution of the problem because there are still other relations to be taken into account, as that between the length of

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the tube and that of the liquid column in it, as well as the range of the shaking movement, and further the relation between the diameter of the tube and the viscosity of the liquid and the velocity of the motor. If the problem is already very complicated in the case of water, it is still more so in that of serum, for a froth will be formed which keeps the air included in its bubbles and so prevents the air in a certain degree from getting through the serum. Especially disturbing is the different absolute amount of complement, which results in varying either the quantity or the concentration of the serum. However, in the case of serum, which is being shaken, the succeeding inactivation offers a certain measure of the intensity of shaking. In order to render it possible to have the absolute amount of complement constant in each tube and at the same time to vary the concentration, I took cylinders of the same diameter and different length but such that the relation of the length to the volume of the liquid was the same in all tubes. But now the relation of the tube-length to the shaking range is varied and renders an exact comparison impossible. It would appear evident that this problem is not capable of exact treatment by experiment unless several shaking apparatus are used at the same time, which allow the shaking distance to be altered, while all other conditions can be kept constant. However, my experiments allow some qualitative conclusions to be drawn and therefore some of them may be reproduced as follows:

Experiments regarding the influence of concentration and of the proportion between volume of tube and volume of liquid on the inactivation by shaking.

EXPERIMENTS I—VI.

EXP. I. Guinea-pig serum $\frac{1}{10}$ diluted is shaken in tubes of 20 c.c. volume 5½ hours at 36°. 160 vibrations in a minute.

[Equal concentration—different quantities of serum.]

	Control	I	II	III	IV	V	VI
	Quantity of $\frac{1}{10}$ serum...	15 c.c.	10 c.c.	8 c.c.	5 c.c.	3 c.c.	2 c.c.
Haemolysis: (1 c.c. $\frac{1}{10}$ compl. + 1 c.c. of sens. red corpuscles. *AB* dose = 0·0025.)	■	■	■	□	□	□	□

Tube I showed no precipitate, tube II only a slight amount, while in all other tubes a thick precipitation could be observed.

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EXP. II. In cylinders of 50 c.c. volume different quantities of $\frac{1}{10}$ diluted guinea-pig serum is shaken 3 hrs. at 36°.

		Control	I	II	III
Quantity of compl. $\frac{1}{10}$ ...			35 c.c.	10 c.c.	5 c.c.
Looking after 3 hrs. shaking...		Clear	Very slight dimness	Dim	Thick precipitate
Haemolysis: (1 c.c. sens. red corp. *AB* dose = 0·0025.)	$\frac{1}{10}$ compl. c.c. 1·0	■	◙	◨	▗
	0·75	■	▟	◲	▗
	0·5	■	◲	▗	□
	0·25	■	▗	▗	□
	0·1	■	□	□	□
	0	□	□	□	□

EXP. III. In cylinder-shaped tubes of 35 c.c. vol. equal quantities (12·7 c.c.) are shaken 5 hours at 35°, the concentration being varied.

		Control	I	II	III	IV	V	VI
Concentration of compl....		$\frac{1}{10}$	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{6}$	$\frac{1}{10}$	$\frac{1}{15}$	$\frac{1}{20}$
$\frac{1}{1}$ complement ser. c.c. ...		1·27	6·35	3·175	2·1	1·27	0·84	0·63
0·85 % NaCl c.c. ...		11·43	6·35	9·525	10·6	11·43	11·86	12·1
Haemolysis after 5 hrs. shaking. (1 c.c. sens. red cells *AB* dose 0·0025 c.c.)	Compl. 0·1	■	■	◙	▟	▟		
	0·05	■	◙	◙	▟	◲	◲	◲
	0·025	▟	◲	◲	◲	▗	▗	▗
	0	□	□	□	□	□	□	□

EXP. IV. Guinea-pig serum was shaken $2\frac{1}{2}$ hours at 36° in tubes of the same length = 15·2 cm., the same diameter = 13 mm. (volume $v = 20·17$ c.c.). The serum was diluted to $\frac{1}{10}$ and the height of the serum standing in the tubes is indicated by h, while the serum quantity is indicated by m.

		Control	I	II	III	IV	V	VI
	h		10·13	7·6	5·06	3·8	3·04	1·52
	$\frac{v}{m}$		1·5	2	3	4	5	10
Haemolysis: (1 c.c. sens. red cells. *AB* dose = 0·0025 c.c.)	$\frac{1}{10}$ compl. c.c. 1·0	■	■	■	■	■	◙	□
	0·75	■	■	■	■	■	▟	□
	0·5	■	■	■	◙	◙	◨	□
	0·25	◙	◲	◲	▗	□	□	□
	0·15	◨	□	□	□	□	□	□
	0·1	□	□	□	□	□	□	□
	0	□	□	□	□	□	□	□

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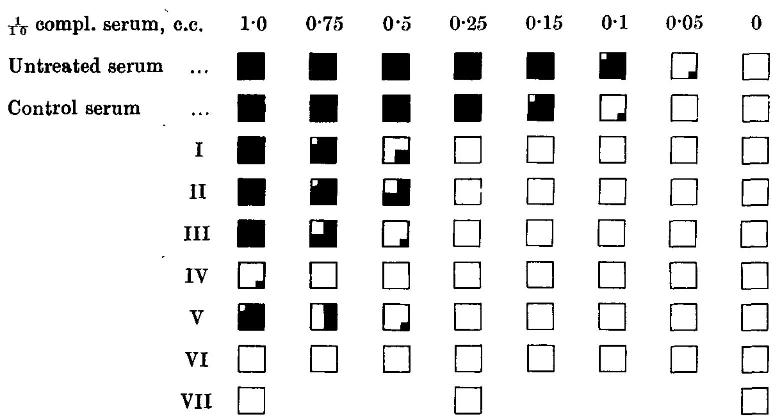
Exp. V. Seven tubes with the following measures

	Diameter	Length	Volume
I	2·7 cm.	17·0	95
II	1·88	17·1	47
III	1·58	16·8	32
IV	1·3	15·2	20
V	1·0	16·7	15
VI	0·85	16·5	9·5
VII	0·68	15·6	5·5

were filled with $\frac{1}{10}$ diluted guinea-pig serum to $\frac{1}{4}$ of their length. After *two* hours shaking in all tubes precipitation occurred, but from tubes I to VII there were an increasing precipitation and a decreasing dimness, so that in tube I the serum was very dim but only a slight precipitate on the bottom, while in tube VII the serum looked quite clear with thick floating coagula.

Haemolysis with 1 c.c. sens. red corpuscles. *AB* dose = 0·0025 c.c.



Tube IV had a different length compared with the other tubes, which may be the reason for the different effect of shaking.

Exp. VI. Fresh complement in different concentration was shaken in different tubes such that the absolute amount of complement was the same in each tube. [34°, 6 hours shaking.]

	I	II	III	IV	V
Tube volume (v)	4	10	16	20	30
Serum quantity (m)	1·6	4·0	6·4	8	12
Concentration	$\frac{1}{2}$	$\frac{1}{5}$	$\frac{1}{8}$	$\frac{1}{10}$	$\frac{1}{15}$
Absolute amount of original compl. ser.	0·8	0·8	0·8	0·8	0·8
$\frac{v}{m}$	2·5	2·5	2·5	2·5	2·5

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After 6 hours shaking, all serum is brought to $\frac{1}{15}$ dilution with saline. Haemolysis with 1 c.c. sens. red corp. *AB* dose = 0·0012 c.c.

$\frac{1}{15}$ compl., c.c.	1·0	0·5	0·25	0·15	0·1	0
Control ...	■	■	■	■	■	□
I	□		□			□
II	◨	◲	□	□	□	□
III	■	■	▟	◲	□	□
IV	■	■	▟	□	□	□
V	■	■	□	□	□	□

Analysis of experiments I–VI.

From these experiments it may be concluded, that a dilution of 1 : 10 gives better results in regard to shaking inactivation than any higher concentration, but in some experiments equally good results were obtained by using dilutions of 1 : 15 and 1 : 20, so that I refrain from speaking of an optimum. With regard to the already mentioned relation between the volume of the tube and the quantity of the shaken serum the best results are obtained if the quantity of the latter is as small as possible compared with the total volume of the tube, but there must be enough serum present to prevent it from becoming entirely converted to froth, which is then no longer exposed to the same intensity of agitation. In another paper (Schmidt, 1913), some remarks were made as to the necessity of taking the froth into consideration in measuring the surface-tension, but so far as complement action is concerned, I did not succeed in tracing any difference in the lytic action of the froth or of the remaining liquid. Generally speaking, the smaller the quantity of shaken serum is, in comparison with the tube volume, the sooner dimness and the formation of coagula occur, which in my opinion represent the first stage of the inactivation. I did not succeed in determining a definite relation between the volumes, for there are so many unforeseen circumstances that in order to explain such differences individual differences in the guinea-pig sera must be presumed.

The precipitate in shaken serum.

Jakoby and Schuetze (1910) observed the formation of precipitates in the shaken serum, which they found to have no direct connection with the complement inactivity. Ritz (1912) wrote in a footnote to his

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paper, that the precipitation runs to some extent parallel with the inactivation but did not exactly correspond with the latter.

On summarising my experiments I can say that I have never seen a serum inactivated by shaking without a precipitate occurring previously, but many sera were observed to be dim and filled with floating coagula. These sera were however not yet inactivated, although corresponding to the intensity of the coagulation they were distinctly weakened in regard to their complement function. I therefore conclude, that the precipitation must in time precede the inactivation, which latter can never take place without the formation of the first. Now the question arises, what that precipitate consists of.

W. Ramsden (1894) showed that shaking of different protein solutions effected a partial separation of the soluble substance in the form of membranes, and that it was possible by means of enlarging the surface of the protein solutions with respect to the gas, as in the process of shaking, to cause almost the total amount of protein of a diluted egg-white solution to coagulate and separate. According to Ramsden (1903, 1904), protein lowers the surface-tension of water and is therefore adsorbed on surfaces and thus relatively concentrated. This increase of concentration in the surface leads to aggregation of particles of protein, before isolated, and further to a diminution of the specific surface of the particles, which is followed by sedimentation due to gravitation and depending upon the viscosity of the solution. The presence of a free surface against gas is necessary to produce this form of coagulation, according to Ramsden (1903, 1904). But the nature of the gas has no influence in so far as it is chemically inactive. With respect to the sera, which are solutions of protein, it is evident, that by shaking, similar phenomena must be observed, especially as the surface tension of serum is lower than that of water and sera possess the tendency to form a relatively high surface viscosity, which enables them to produce stable froth. Now shaking produces also in sera aggregation of proteins and coagulation, which leads to a partial separation of the protein in the form of a precipitate. After removing the precipitate and shaking again a new precipitate is formed. In another paper (Schmidt, 1913) I was able to show that during this procedure the surface tension is not lessened as in the case of thermoinactivation and coagulation by heat.

It appears evident therefore that the precipitate produced by mechanical agitation represents nothing else than mechanically coagulated protein. Ramsden also observed the formation of coagula in shaken

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sera, but he noticed that most of them went into solution again, only a part of the coagula remaining insoluble. It takes therefore much longer time to produce a mechanical coagulation in sera than in solutions of egg-white. According to Ramsden the coagula thus produced in sera consist of unaltered serum albumin. With regard to the relation of this precipitate to complement, Jakoby and Schuetze state that they observed a reactivation of such precipitate, although in a slight degree, by each of the two complement fractions. Ritz (1912) could not confirm this statement and I too must agree with Ritz. I found the precipitate to be insoluble either in 0·85 % saline solution or in distilled water or in serum, even if heated to 56°. But the precipitate disappears from the eye by adding some KOH, a phenomenon which, according to Ramsden, does not imply solution of the precipitate, which is merely soaked. I found neither end-piece nor mid-piece to be activated by the precipitate, even in the slightest degree. I am inclined to identify this precipitate with that, which is often seen in sera, which is kept in a cold room for a long time and which I have found to be also insoluble even by heating to 56°.

Influence of the nature of the gas on serum.

Provided that the gas in which the serum is shaken is chemically inactive its nature does not play any part, according to Ramsden. The latter shook protein solutions also *in vacuo* and obtained the same phenomenon of coagulation. But recently Courmont and Dufour (1912 *b*) published experiments, in which they claim to have obtained the shaking inactivation of complement-containing sera much quicker, if the serum has been shaken in oxygen, whereas no alteration of the complement function took place, if the serum was shaken in nitrogen or *in vacuo*. The authors believe it to be very probable that the inactivation by shaking in air is of the nature of an oxidation process. In order to examine, how far complement action is influenced by the presence of oxygen I undertook a series of different experiments. First of all I studied the influence of oxygen on thermoinactivation.

(*a*) *The influence of oxygen on inactivation by heat.*

For this purpose serum, undiluted as well as diluted to 1 : 10 by saline solution, was evacuated and then the vacuum filled with hydrogen. After shaking the serum several times in hydrogen it was again evacuated and then again hydrogen allowed to enter. This procedure was

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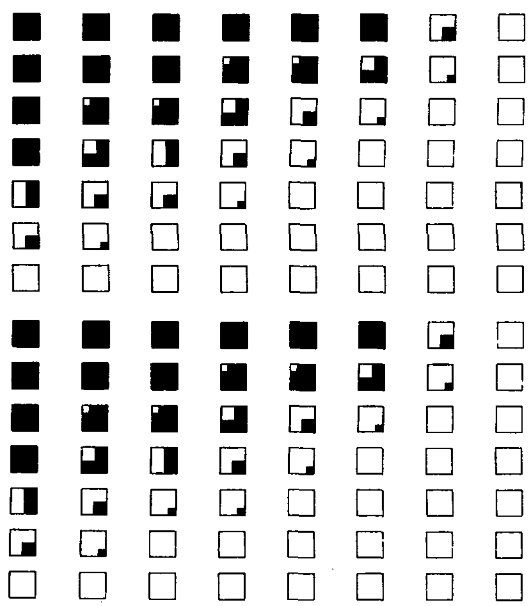
repeated seven times and finally the serum was sealed in an atmosphere of pure hydrogen. In can confirm Simnitzki's (1903) statement, that the removal of oxygen does not influence the complement action, the haemolysing effect being the same as before. As control to this procedure the same serum was saturated with pure oxygen by shaking it repeatedly in the gas and then sealing it in a glass tube containing pure oxygen. The sera treated in this way were exposed to the temperature of 56°, the time being varied. The result of the thermoinactivation thus produced can be seen from the following table:

The influence of oxygen on thermoinactivation.

Exp. VII. Each tube, containing 1 c.c. complement serum, was exposed to heat of 56° during different times, under H and O. Haemolysis with 1 c.c. sens. red corp. *AB* dose $=0{\cdot}0025$ c.c., the total volume in each tube being 2 c.c.

Compl. serum $\frac{1}{10}$ c.c. ... 1·0 0·75 0·5 0·25 0·15 0·1 0·05 0

Time of exposure to 56°, mins. ... 0, 1, 2, 3, 4, 5, 6; 0, 1, 2, 3, 4, 5, 6



The table shows that not the slightest difference in the effect of heat on complement-containing serum resulted whether the serum was exposed to heat under much oxygen or without oxygen. In an astonishingly short time (cf. Husler (1912), H. Schmidt (1913)) the serum was inactivated in both cases.

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(b) *The influence of oxygen on the storage of complement.*

I made further investigations as to the influence of oxygen in inactivating complement which had been stored. For this purpose I treated sera as above mentioned and kept them under oxygen, nitrogen and hydrogen for days and weeks, in most cases in the cold room but sometimes at room temperature and at 37°. In some experiments I allowed oxygen and hydrogen to blow continuously over the serum for 24 hours at 37°. I can give a brief summary of all these experiments by saying that I never succeeded in demonstrating unequivocally, that complement can be preserved longer in the absence of oxygen than with it. In experiments, which seem to give this result, larger bacterial growth could be observed in the serum kept under oxygen. Such sera produced a certain violet colour in all test-tubes whether haemolysis occurred or not. This phenomenon is due to bacterial growth, according to Kostrzewski (1911). K. Hara (1913) showed that complement can be preserved longer if kept sterile and that bacterial growth causes the disappearance of the complement and the anticomplementary property, which can be observed in some sera. I succeeded however in keeping the complement-containing serum in a slight degree of activity for about four weeks but I could not find the complement to be preserved longer in the absence of oxygen. In fact no difference could be traced.

(c) *The influence of oxygen on inactivation by shaking.*

Special investigations were then made in regard to the inactivation by means of shaking. Courmont and Dufour shook complement-containing serum *in vacuo*, giving 6 mm. Hg pressure. I refrained from using a vacuum, because in an even stronger vacuum of air as these authors used, enough molecules of oxygen are still present to effect eventually an oxidation, and I appeal to the fact that Ramsden saw coagulation occurring in protein solutions, which were shaken *in vacuo*. I shook the sera in different gases as oxygen, hydrogen and nitrogen at the same time varying the conditions with regard to concentration and relation between the volumes of the tube and of the serum. In the case of hydrogen and nitrogen employed care was taken to ensure complete removal of all oxygen. I can also here summarise my results by saying that I observed in every case the formation of coagula and that the greater the attention paid to render the external conditions as constant as possible, the more the results—in the beginning often found to be contradictory—became the same. In the earlier experiments I often

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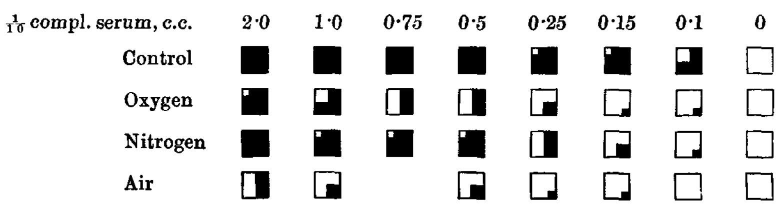
obtained confirmation of the experimental results of Courmont and Dufour, that is to say, destruction of complement, if it is shaken in oxygen and only a slight alteration of the activity when shaken in hydrogen or nitrogen. But sometimes I found the contrary, complete destruction in nitrogen or hydrogen and almost none in oxygen. But the more the conditions, under which the sera were shaken, were rendered equal, the less was the difference in the degree of inactivity obtained, and finally no more difference could be observed in the haemolysis produced by complement-containing serum whether shaken in oxygen or in hydrogen or nitrogen.

I give in the following a few of my experiments:

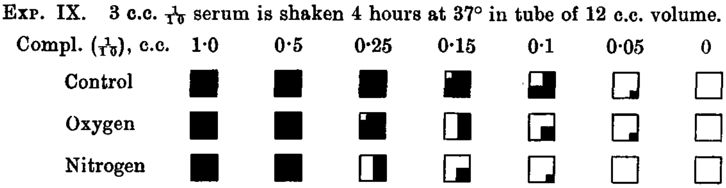
Experiments showing the influence of the nature of the gas on inactivation by shaking.

Experiments VIII—XI.

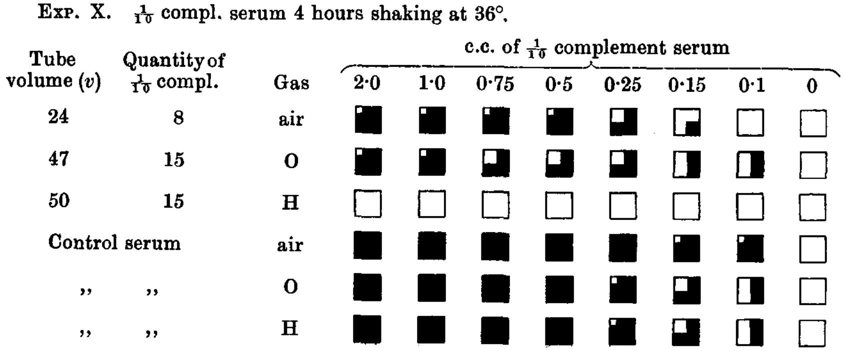
Exp. VIII. Fresh complement serum in $\frac{1}{10}$ dilution was shaken for 6 hours at 36°. The relation between the tube volume (35 c.c.) and the serum quantity was kept constant = 2. Haemolysis with 1 c.c. sens. red corp. *AB* dose = 0·0025 c.c.



Exp. IX. 3 c.c. $\frac{1}{10}$ serum is shaken 4 hours at 37° in tube of 12 c.c. volume.



Exp. X. $\frac{1}{10}$ compl. serum 4 hours shaking at 36°.



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EXP. XI. $\frac{1}{10}$ complement serum is shaken under conditions as equal as possible. Tube volume 20 c.c. Complement serum quantity 8·5 c.c.

$\frac{1}{10}$ compl. ser., c.c.	2·0	1·5	1·0	0·75	0·5	0·25	0·15	0·1	0	Time of shaking
O	■	■	■	■	■	■	▟	◲	□	1 hour
H	■	■	■	■	■	■	▟	◲	□	1 hour
O	■	■	■	■	■	▟	◨	◲	□	2 hours
H	■	■	■	■	■	▟	◨	◲	□	2 hours
O	■	■	■	◨	◲	◲	□	□	□	3 hours
H	■	■	■	◨	◲	◲	□	□	□	3 hours

Courmont and Dufour (1912 *b*) also showed a slight loss of complement action occurring in the sera shaken *in vacuo* or with nitrogen in comparison with the control. I am inclined to consider the results obtained by these authors in regard to shaking inactivation as experimental errors caused by slight alterations in the external conditions but sufficient to render the intensity of the shaking agitation different in each case. That small alterations in the relation between the volumes of the tube and the liquid are still capable of giving variant results is shown by Ritz (1912), and I too had plenty of opportunity of being convinced of these facts (cf. Exp. X).

Now this result agrees with that obtained by Ramsden (1904), who said that the nature of the gas is of no importance to the mechanical coagulation in protein solutions. However, as a sort of *experimentum crucis* I mention the following experiment:

After removing the oxygen as completely as possible from a serum by a procedure similar to that above mentioned, I added carefully cleaned glass beads and closed the tube with a rubber stopper, so that no trace of free gas could be observed even after shaking the tube for many hours. An intense shaking giving 200 vibrations in the minute caused whirlpools in the liquid by the movement of the glass beads. The mechanical agitation thus produced is relatively small compared with the shaking in gases. However, the experiment shows that the complement action was weakened and after shaking a very long time at 36° C. complement was rendered practically inactive, the serum being then very dim owing to the presence of coagulated proteins.

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Complement-containing serum shaken with glass beads without any gas.

Exp. XII. $\frac{1}{10}$ serum is shaken for 3 hours at 36°. Tube volume = 47 c.c. The tubes with air, O and H were filled with 12 c.c. $\frac{1}{10}$ complement serum. One tube was partially filled with glass beads and then filled with serum without gas.

c.c. of $\frac{1}{10}$ compl.	1·0	0·75	0·5	0·25	0·15	0·1	0·05	0
Shaken in air ...	□	□	□	□	□	□	□	□
Shaken in oxygen ...	□	□	□	□	□	□	□	□
Shaken in hydrogen ...	□	□	□	□	□	□	□	□
Shaken with glass beads	■	■	■	◨	□	□	□	□
Control, in air ...	■	■	■	■	■	◲	□	□
Control, in oxygen ...	■	■	■	■	■	◲	□	□
Control, in hydrogen ...	■	■	■	■	■	◲	□	□

$\frac{1}{10}$ complement serum, 8 hours shaken with glass beads at 36°.

$\frac{1}{10}$ compl. ser. c.c.	1·0	0·5	0·25	0·15	0·1	0
Normal serum ...	■	■	■	■	◨	□
Control serum ...	■	■	◨	◲	◲	□
Shaken with glass beads	◲	□	□	□	□	□

From these experiments it appears to be evident that inactivity by shaking is due to mechanical influence, and that every factor capable of increasing the intensity of agitation has an accelerating effect upon the destruction of complement by means of agitation. To presume an oxidation by the shaking with air is not necessary and is not in agreement with the observed facts. Shaklee and Meltzer (1909) found also in shaking proteolytic ferments "that the destruction was not due to an oxidation by the oxygen in the air," which was proved by the fact "that the destructive effect remained the same, when the space above the liquid within the bottle was filled with hydrogen."

Reactivation of serum inactivated by shaking *by normal complement and its fractions.*

It has been shown that the shaking inactivation is associated with a mechanical coagulation and separation of a part of the proteins, and further these coagulated proteins are proved to have no direct connection with the complement. Now the shaken inactivated complement is represented by the supernatant fluid of the centrifugalised shaken

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serum. I mentioned above that Jakoby and Schuetze (1910) found that fresh end-piece can reactivate this fluid to a large extent, a statement which has been confirmed by Ritz (1912) and Kashiwabara (1913), who further succeeded in reactivating shaken inactive serum by thermoinactive serum. In order to prove these different possibilities of reactivating a shaken inactive serum I undertook some experiments and was able to obtain more or less the same results.

I give a brief record of some of my experiments in the following:

Experiments showing the reactivation of complement inactivated by shaking, by normal complement and its fractions.

EXP. XIII. Normal end-piece and mid-piece are obtained by the procedure of Sachs and Altmann and are each in $\frac{1}{10}$ dilution so that 1·0 c.c. end-piece plus 1·0 c.c. mid-piece correspond with 1·0 of $\frac{1}{10}$ active complement.

	Haemolysis
1·0 c.c. n. end-piece + 1·0 c.c. sensit. red corp. + 1·0 c.c. saline—0·85 %	□
1·0 c.c. n. mid-piece + 1·0 c.c. ,, ,, + 1·0 c.c. ,, ...	□
1·0 c.c. n. mid-piece + 1·0 c.c. ,, ,, + 1·0 c.c. end-piece	■
Shaking precipitate + 1·0 c.c. end-piece + 1·0 c.c. sensit. red corp.	□
,, ,, + 1·0 c.c. mid-piece + 1·0 c.c. ,, ,, ...	□
,, ,, + 1·0 c.c. n. $\frac{1}{10}$ complement + 1·0 c.c. sensit. red corp. ...	■
Centrifugalised shaken serum + 1·0 c.c. n. end-piece + 1·0 c.c. sensit. red corp. ...	■
,, ,, ,, + 1·0 c.c. n. mid-piece + 1·0 c.c. ,, ,, ...	□
,, ,, ,, + 1·0 c.c. $\frac{1}{10}$ n. complement + 1·0 c.c. sensit. red corp.	■
1·0 c.c. $\frac{1}{10}$ complement + 1·0 c.c. end-piece + 1·0 c.c. sensit. red corp.	■
1·0 c.c. ,, ,, + 1·0 c.c. mid-piece + 1·0 c.c. ,, ,, ...	□

These experiments permit one to make the following statement:

After splitting a fresh complement-containing serum successfully into its two fractions by the method of Sachs and Altmann, I was able to confirm the statement that the supernatant fluid of the centrifugalised shaken inactive serum could be brought to full complement action by adding fresh normal end-piece, but no reactivation occurred by addition of mid-piece. A normal haemolytic system is not inhibited by the presence of shaken inactivated serum, as happens occasionally with thermoinactive serum. I mention the confirmation of this statement, made also by Courmont and Dufour (1912 *a*), because it is remarkable

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that the centrifugalised shaken inactive serum had no anticomplementary power in all my experiments, although it acts by its reactivation with end-piece as mid-piece, which latter has sometimes strong anticomplementary action (P. Schmidt (1911), Ledingham and Dean (1912)).

Reactivation of thermoinactive serum.

To examine further the reactivation of a shaken inactive serum by a thermoinactivated serum I investigated first, how far the latter can be reactivated either by fresh serum or by the complement fractions.

Exp. XIV. $\frac{1}{10}$ complement serum has been inactivated by heating it to 55° during 2, 3, 5, 30 mins. By splitting complement serum the following was obtained:

From normal complement serum	n. end-piece and n. mid-piece.
,, thermoinactive complement serum (5 mins. 55°)	end-piece II and mid-piece II.
,, ,, ,, ,, (30 mins. 55°)	end-piece III and mid-piece III.

In the following haemolysing tests always 1 c.c. sens. red corpuscles is taken, the *AB* dose being 0·0025 c.c.

	Haemolysis
1·0 c.c. thermoinactive serum (2 mins. 55°) + 1·0 c.c. 0·85 °/$_0$ NaCl ...	■
1·0 c.c. ,, ,, (3 mins. 55°) + 1·0 c.c. ,, ,, ...	◪
1·0 c.c. ,, ,, (5 mins. 55°) + 1·0 c.c. ,, ,, ...	□
1·0 c.c. ,, ,, (30 mins. 55°) + 1·0 c.c. ,, ,, ...	□
1·0 c.c. thermoinactive serum (2 mins. 55°) + 1·0 c.c. $\frac{1}{10}$ active complement	■
1·0 c.c. ,, ,, (3 mins. 55°) + 1·0 c.c. ,, ,, ,,	■
1·0 c.c. ,, ,, (5 mins. 55°) + 1·0 c.c. ,, ,, ,,	■
1·0 c.c. ,, ,, (30 mins. 55°) + 1·0 c.c. ,, ,, ,,	◨
1·0 c.c. n. end-piece + 1·0 c.c. 0·85 °/$_0$ NaCl	□
1·0 c.c. n. mid-piece + 1·0 c.c. ,, ,, ...	□
1·0 c.c. n. end-piece + 1·0 c.c. n. mid-piece	■
1·0 c.c. end-piece II + 1·0 c.c. 0·85 °/$_0$ NaCl	□
1·0 c.c. mid-piece II + 1·0 c.c. ,, ,, ...	□
1·0 c.c. end-piece II + 1·0 c.c. mid-piece II	■
1·0 c.c. end-piece III + 1·0 c.c. 0·85 °/$_0$ NaCl	□
1·0 c.c. mid-piece III + 1·0 c.c. ,, ,, ...	□
1·0 c.c. end-piece III + 1·0 c.c. mid-piece III	□

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1·0 c.c. n. end-piece + 1·0 c.c. mid-piece II	■
1·0 c.c. n. end-piece + 1·0 c.c. mid-piece III	⊡
1·0 c.c. n. mid-piece + 1·0 c.c. end-piece II	□
1·0 c.c. n. mid-piece + 1·0 c.c. end-piece III	□
1·0 c.c. thermoinactive serum (2 mins. 55°) + 1·0 c.c. n. end-piece ...	■
1·0 c.c. ,, ,, (3 mins. 55°) + 1·0 c.c. ,, ...	■
1·0 c.c. ,, ,, (5 mins. 55°) + 1·0 c.c. ,, ...	■
1·0 c.c. ,, ,, (5 mins. 55°) + 1·0 c.c. end-piece II ...	◨
1·0 c.c. ,, ,, (5 mins. 55°) + 1·0 c.c. end-piece III ...	◲
1·0 c.c. ,, ,, (30 mins. 55°) + 1·0 c.c. n. end-piece ...	□
1·0 c.c. ,, ,, (30 mins. 55°) + 1·0 c.c. end-piece II ...	□
1·0 c.c. ,, ,, (30 mins. 55°) + 1·0 c.c. end-piece III ...	□
1·0 c.c. thermoinactive serum (2 mins. 55°) + 1·0 c.c. n. mid-piece ...	□
1·0 c.c. ,, ,, (3 mins. 55°) + 1·0 c.c. ,, ...	□
1·0 c.c. ,, ,, (5 mins. 55°) + 1·0 c.c. ,, ...	□
1·0 c.c. ,, ,, (30 mins. 55°) + 1·0 c.c. ,, ...	□

Analysing these experiments one may conclude:

Thermoinactive serum can be reactivated by adding fresh active serum (cf. H. Schmidt, 1913). The quantity of active serum necessary to produce an haemolytic effect on sensitized red cells in combination with the thermoinactive serum depends upon the anticomplementary property of the latter, which is greater the longer the serum is exposed to 56°. The reactivation of complement action by the isolated fractions depends also upon the intensity of the exposure to heat. In thermo-inactivation first the end-piece and then the mid-piece is destroyed (Sachs, 1913), and in guinea-pig serum inactivated by the usual way (30 minutes, 56°) no mid-piece is to be traced (Husler (1912)). In agreement with this statement guinea-pig serum only a short time—5 minutes—exposed to heat, long enough to render the serum inactive, can be reactivated by splitting it and mixing the two fractions again, as was also found by Mutermilch (1911). But this phenomenon is not obtained if the time, during which the serum is heated, has been longer. If the treatment by heat is too long, say 30 mins. or longer, it loses its property of being reactivated by end-piece, but the thermostability of the complement fractions seems to vary in different guinea-pigs.

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Reactivation of shaken serum by thermoinactive serum.

With regard to the reactivation of shaken inactive serum by thermoinactive serum I give the following experiments:

Exp. XV. The following haemolysing tests were made with 1·0 c.c. sensit. red cells, the *AB* dose being 0·0025 c.c.

		Haemolysis
1·0 c.c. shaken inactive serum	+ 1·0 c.c. $\frac{1}{10}$ complement	■
1·0 c.c. ,, ,,	+ 1·0 c.c. n. end-piece	■
1·0 c.c. ,, ,,	+ 1·0 c.c. thermoinactive ser. (2 mins. 55°)	■
1·0 c.c. ,, ,,	+ 1·0 c.c. ,, ,, (3 mins. 55°)	■
1·0 c.c. ,, ,,	+ 1·0 c.c. ,, ,, (5 mins. 55°)	■
1·0 c.c. ,, ,,	+ 1·0 c.c. ,, ,, (30 mins. 55°)	⊡
1·0 c.c. control serum	+ 1·0 c.c. ,, ,, (2 mins. 55°)	■
1·0 c.c. ,, ,,	+ 1·0 c.c. ,, ,, (3 mins. 55°)	■
1·0 c.c. ,, ,,	+ 1·0 c.c. ,, ,, (5 mins. 55°)	◨
1·0 c.c. ,, ,,	+ 1·0 c.c. ,, ,, (30 mins. 55°)	◪
1·0 c.c. shaken inactive serum	+ 1·0 c.c. ,, ,, (2 mins. 55°) 2 hrs. shaken	■
1·0 c.c. ,, ,,	+ 1·0 c.c. ,, ,, (5 mins. 55°) ,, ,,	◲
1·0 c.c. ,, ,,	+ 1·0 c.c. ,, ,, (30 mins. 55°) ,, ,,	□
1·0 c.c. ,, ,,	+ 1·0 c.c. NaCl	⊡

From this experiment it appears that shaken inactive serum can be activated by thermoinactive serum but only if the latter has not been exposed to heat for too long a time, so there is still some end-piece left active. I am inclined to render the activation by thermoinactive serum equal to that obtained by normal end-piece. Kashiwabara (1913) did not always succeed in obtaining the reactivation by thermoinactive serum probably because the time he used for the thermoinactivation had been too long, so that the end-piece was destroyed completely. I saw in some experiments a reactivation of shaken inactive serum produced by serum which had been heated half an hour to 56°, but here neither the reactivation nor the inactivity by shaking had been complete. Finally I was able to confirm the statement of Kashiwabara (1913) that thermoinactive serum loses the property to reactivate shaken inactive serum, if it has been shaken before. In shaking thermoinactive serum

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the observation can be made that much longer time is required to produce mechanical coagulation. It may be that this fact stands in some relation to the statement of Mutermilch (1911), viz. that it takes a much longer time to precipitate the serum globulin by dialysis in heated sera than in normal sera.

Possible explanation of the inactivation by shaking.

Now is it possible to give a reasonable explanation of the nature of the shaking inactivation? Presuming the complement to be of the nature of a ferment the shaking inactivation of complement-containing serum can be placed in some relation with the inactivation by shaking of pepsin and trypsin (Shaklee and Meltzer, 1909) and of the lab-ferment (Schmidt-Nielsen, 1909). P. Schmidt (1911, 1912) showed that active complement is rendered inactive, if it is kept in contact with an emulsion of globulin, prepared from a thermoinactive serum, during 25–30 minutes at 37°. He supposes an adsorption, which increases with the time. Further, he showed that only an overplus of immune bodies, containing albumin, is able to set free the complement from the globulin surface. It is further shown by Landsteiner and Stankovic (1906) that coagulated serum protein is able to adsorb complement from a serum, thus rendering the serum inactive. In agreement with these facts it may be possible, that the coagulated serum proteins produced by the agitation adsorb the complement and that this adsorption increases with the time, thus explaining the observations of Ritz that a serum shaken for a long time loses its property of being reactivated by end-piece.

However, I was unable to obtain a loss of complement action when I brought fresh $\frac{1}{10}$ diluted complement-containing serum in contact with the precipitate, but this negative result may be due to an alteration of the degree of dispersity occurring in shaken sera.

In any way, the fact would be difficult to explain, that the isolated precipitate can by no means be reactivated while the supernatant inactive fluid can be reactivated by end-piece, if the complement were bound to the separated precipitate.

In view of these difficulties I must confess to be unable to give any satisfactory explanation of what takes place in the process of inactivation by shaking.

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General Conclusions.

1. Guinea-pig serum can be inactivated by shaking, the process being generally the quicker the higher the temperature.

2. It is advantageous for the purpose of inactivation by shaking to dilute the serum to 1:10 with saline solution and to take as small a quantity in comparison with the volume, in which it is to be shaken, as the resultant froth formation will permit.

3. In every serum a coagulation of a part of the proteins occurs owing to the agitation.

4. This precipitate always precedes the inactivation in time but no direct connection exists between the constituents of the precipitate and the complement.

5. From the observations, viz. that oxygen does not influence either the effect of heat on complement or the period of its survival in storage or the inactivation by mechanical agitation, it follows, that no oxidation occurs during inactivation by shaking but that the latter is independent of the nature of the gas.

6. The precipitate in shaken sera cannot be reactivated either by mid- or end-piece.

7. The supernatant fluid can be reactivated by fresh complement, by normal end-piece and by thermoinactive serum. Thermoinactive serum can however reactivate shaken inactive complement, only if it still contains active end-piece.

8. If thermoinactive serum is shaken it loses its property of reactivating shaken inactive serum.

9. No satisfactory explanation of the inactivation by shaking can be adduced.

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