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THE MECHANISM OF THE AGGLUTINATION REACTION.

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SINCE Gruber and Nicolle expressed the opinion that the agglutination of bacteria by specific immune sera was due to the surfaces of the bacteria becoming viscous under the influence of the specific sera, many theories have been advanced aiming at a more complete explanation of the reaction. Bordet (1899) considered that the agglutinins by uniting with the agglutinable substances lead to changes in molecular attraction between the elements affected, either as among themselves, or between them and the surrounding fluid. He distinguished the two phases of the reaction, (1) the interaction between the agglutinin and the bacteria, and (2) the agglomeration of the bacteria.

Kraus (1897) observed that the addition of specific agglutinating sera to bacterium-free filtrates of broth cultures of cholera vibrios, typhoid and plague bacilli resulted in the formation of a precipitate, and according to Nicolle (1898) watery extracts of agar cultures of *B. typhosus*, *B. coli* and *V. cholerae* gave a precipitate with the appropriate antiserum. He showed further that even non-specific bacteria, added to a mixture of antiserum and extract of homologous bacteria, were completely agglutinated. In these experiments, however, he did not use high dilutions of either serum or extract.

Paltauf (1897) based his theory of agglutination on these observations. According to him the agglutination of bacteria by a specific serum is due to the formation of a coagulum outside the bacteria. This coagulum, which is the result of the interaction of agglutinin and agglutinable substance diffused from the bacterial cells, draws the bacteria together mechanically.

Löwit (1903) and later Arkwright (1914) claim to have demonstrated a coagulum around agglutinated bacteria, but a number of other workers have been unable to confirm these observations.

Recently Arkwright (1914) has brought forward further evidence in favour of Paltauf's hypothesis. He found that if a mixture were made of extract of typhoid bacilli in distilled water, typhoid immune serum and non-specific bacteria, such as *B. coli* or *B. acidi lactici*, or even particles of animal charcoal or kieselguhr, the bacteria or inorganic particles were agglutinated with very considerable dilutions of the antiserum and extract. Thus in a mixture of extract of typhoid bacilli, typhoid immune serum and *B. coli* he obtained definite macroscopic agglutination of the *B. coli* with a dilution of 1 : 27,000 serum and 1 : 96 extract. Control experiments showed that in these dilutions no visible precipitate was formed from the interaction of the serum and extract alone, and the *B. coli* were not agglutinated by the serum alone. If a mixture of extract and antiserum in certain concentrations were made, a visible precipitate was formed; the addition of normal serum in certain proportions to such a mixture increased the bulk of the precipitate.

Previously Dean (1912) had shown that the addition of a small quantity of a solution of euglobulin—that part of the serum proteins which is precipitated by slightly acidifying a dilution of serum in distilled water—to a mixture of horse serum, increased the quantity of the precipitate, and there was visible precipitation with higher dilutions of antiserum in the presence of the euglobulin than in its absence. Further, specific antiserum caused agglutination of bacteria in higher dilutions when a small quantity of euglobulin solution, or of normal serum, was added.

Moreschi (1908) found that the addition of homologous precipitating antiserum to a mixture of bacteria and specific agglutinating serum increased the agglutination titre of the specific serum to a greater degree than the addition of normal serum. Thus in one experiment using human typhoid immune serum he found that a dilution of 1 in 40 gave agglutination with *B. typhosus* with the addition of 0·05 of normal rabbit serum, and 1 : 1280 gave agglutination with the addition of 0·05 of antihuman rabbit serum.

These observations suggest strongly that the agglutination of bacteria by a specific immune serum is due to the formation of a precipitate outside the bacteria, which in some way causes the bacteria to clump together and become agglomerated.

The experiments of Kraus, of Nicolle and of Arkwright show that a precipitate is formed as the result of the interaction between bacterial extract and specific immune serum and that this precipitate will cause the agglomeration of non-specific bacteria.

In Moreschi's experiments a precipitate is formed in the interacting mixture as a result of the interaction between the human serum and the antihuman serum, and it seems justifiable to assume that it is this precipitate which increases the agglutinating titre of the human typhoid immune serum.

The result of Dean's experiments may be correlated with those of Arkwright, and again it seems justifiable to assume that the increase in titre of the agglutinating serum is due to the increased formation of precipitate.

The conception that the agglutination of bacteria is brought about by the formation of a precipitate is strengthened by the observations of Scheller (1910). He found that if a mixture of bacteria and specific immune serum were shaken shortly after the commencement of the reaction, the agglutination of the bacteria might be completely inhibited.

He considered that the disagglutination was due to the rendering homogeneous of the precipitate which he supposed to be the cause of the agglutination.

It has for a long time been recognised that the agglutination reaction and the precipitin reaction present many analogies.

The agglutinins as well as the precipitins show the phenomenon of inactivation and the production of an inhibitory property on heating at certain temperatures.

In both the precipitin and agglutination reactions, as Bordet pointed out, the presence of sodium chloride, or at any rate of an electrolyte, is necessary for the agglutination.

Both precipitins and agglutinins are intimately connected with the globulin fraction of the serum, and are carried down completely when the globulins are precipitated by magnesium sulphate or ammonium sulphate. Further the denaturation of the globulin is accompanied by complete destruction both of agglutinin and precipitin.

In the precipitin reaction Welsh and Chapman (1906) have shown that the greater part, if not the whole of the precipitate, is derived from the antiserum and not from the antigen as previously believed, and that antigen is not removed in appreciable amounts from the mixture of antigen and antiserum by the precipitate. They attempted to exhaust the antigen in solution by the repeated addition of antiserum, and found that the sixth successive addition gave as much precipitate as the first, although the amount of antigen could not, on account of the dilution, be more than one-tenth of that originally present, assuming that none of it was removed in the precipitate.

I have found that bacteria and specific agglutinating sera gave analogous results. A thick emulsion of *B. typhosus* in normal saline solution was mixed with typhoid goat antiserum, so that the dilution of antiserum was 1 : 10. After twenty-four hours the agglutinated bacteria were centrifuged off completely and the supernatant fluid removed. A further quantity of antiserum was added to the superfluid and after twenty-four hours there was a large precipitate which was separated. This process was repeated four times and there was still a marked precipitate after the fourth addition of antiserum.

This experiment was repeated a number of times with immune sera from different animals. Microscopical examination of the precipitate showed it to consist of aggregates of amorphous material. From this it is evident that the antigen was not completely removed with the agglutinated bacteria, but that part of the antigen had diffused into the surrounding fluid and that the precipitate was largely, if not entirely, derived from the serum added.

There are, however, apparent differences between the two reactions. There is, for example, not complete agreement between the phenomenon of agglutination of bacteria, and that of the production of a precipitate with filtered broth cultures of the same bacteria, by specific sera. Numerous examples have been given by different workers where the formation of agglutinins in a serum has not run parallel with the formation of bacterio-precipitins for the broth cultures of the bacteria.

In my opinion this discrepancy may be explained in the following manner:

While it is undoubted that a precipitate is formed outside the bacterial bodies in an agglutination reaction, it is probable that as bacteria absorb protein from serum, precipitation takes place also within the cells, or at any rate in extremely close relationship to the cell walls.

Joos (1903) and others have shown that agglutinin and agglutinogen are not uniform substances but consist of a number of different components. It seems, therefore, likely that the antigen for one or more components of the agglutinins is retained within the bacteria, because non-diffusible, and in consequence the interaction of antigen and antibody will, in part, take place within the bacterial cell or in the cell wall. Moreover it is not likely that antibodies to different parts of the cell protoplasm are formed at different rates so that serum taken from an animal which is being immunised may at one time give a sufficient precipitate with the antigen retained in the bacterial cell and not with

the diffusible antigen, while at another time it may give a marked precipitate with both.

That the formation of a precipitate within the bacterial cell can give rise to the agglutination of bacteria is shown by the experiments of Neisser and Friedemann (1904). These authors treated bacteria with lead salts, then washed them till the washings contained no trace of lead. The addition of sulphuretted hydrogen water caused immediate strong agglutination and the bacteria were stained black.

Another difficulty which has been advanced in connection with the conception of the identity of the agglutination and precipitin reactions, is the observation that agglutinating sera may produce agglutination in extremely high dilutions while precipitin sera only produce visible precipitates if a relatively large amount of the antiserum be employed.

In a mixture of antigen and a relatively small amount of precipitin serum, the individual molecules of serum protein are widely separated so that the formation of aggregates of sufficient size to be visible may be impossible, besides, the amount of serum protein present may be too small to be visible on separation. The close analogy which exists between the precipitin and agglutination reactions suggests that the agglutination is due to the precipitation of a certain fraction of the serum proteins in intimate relationship to the bacteria. The relatively great size of the bacterium serum protein complex, however, affords ample opportunity for the particles of serum protein to approach one another, and with the bacteria to form aggregates of sufficient size to produce a visible precipitate.

RELATION OF AGGLUTINATION TO THE GLOBULIN OF THE SERUM.

Franceschalli (1909) has shown that in a particular series of precipitin reactions 42·8 per cent. of the globulin of the antiserum was removed in the precipitate.

Dean (1912) has shown that the addition of euglobulin solution to a mixture of bacteria and antiserum increases the titre of the antiserum considerably; in an analogous way the amount of precipitate in a precipitin reaction is increased by the addition of euglobulin solution. He considers that the interaction of the antigen and antibody causes an aggregation of the molecules of a non-specific substance, which is possibly serum globulin, and that this aggregation is an essential part of the process of agglutination.

It seems probable that the greater part at least of the precipitate in a precipitin reaction consists of serum globulin, derived from the antiserum, which is altered in some way and is not necessarily specific.

From analogy, the precipitate which causes the agglutination reaction may also consist of altered globulin with or without some other altered serum proteins, hence agglutinated bacteria should show similar properties to altered or "denaturated" proteins. That this is the case is shown in part by Tullock's (1914) experiments. He agglutinated *B. paratyphosus* B with specific antiserum, washed them twice with distilled water and resuspended in distilled water. A considerably smaller amount of a divalent salt such as calcium chloride or barium chloride than of a monovalent salt such as sodium chloride was needed to produce reagglutination. Chick and Martin (1912) obtained similar results with denaturated serum proteins and denaturated egg albumin in faintly alkaline dispersions.

In the case of acid dispersions of washed agglutinated bacteria Tullock found that the agglutinating value of a salt depended on the valency of the anion as did Chick and Martin in the agglutination of acid dispersions of denaturated proteins; thus sodium sulphate was more active than sodium chloride or barium chloride.

It seems probable, then, that the agglutination of bacteria by specific antiserum is, in the main, due to the formation of altered serum globulin in and around the bacteria and the subsequent flocculation by electrolytes of this altered globulin together with the entangled bacteria.

Effects of Acids and Alkalis on Agglutination.

The effects of low concentrations of acids and alkalis on denaturated proteins have been studied in some detail by several workers, notably Chick and Martin.

(*a*) *Effects of dilute acids and alkalis on the agglutination of bacteria by specific sera.*

If acids and alkalis be added, in sufficient amount, to a mixture of bacteria and specific agglutinating serum, the agglutination of the bacteria is inhibited. Alkalis have a somewhat more marked effect, in this direction, than have acids, as will be observed in Table I, where 0·10 cc. of N/100 potassium hydrate produced some inhibition, while the same amount of N/100 sulphuric acid was without effect.

TABLE I.

Goat *v.* typhoid serum diluted 1 : 10 in normal saline solution 0·1 cc.
Emulsion of 24 hours agar culture of *B. typhosus* in normal saline solution 0·5 cc.
4 per cent. sodium chloride solution 0·1 cc.
N/100 H_2SO_4 or N/100 KOH in each tube in amount indicated.
Contents of all tubes made up to 1 cc. with distilled water.
2 hours at 37° C. 18 hours at room temperature.

	0	·02	·04	·06	·08	·10	·12	·14	·16	·18	·20	·22
N/100 H_2SO_4	+++	+++	+++	+++	+++	+++	+++	++	+	–	–	–
N/100 KOH	+++	+++	+++	+++	+++	++	++	+	+	–	–	–

(*b*) *Effect of the nature of the acid.*

TABLE II.

Rabbit *v.* typhoid serum 1 : 10 in saline, 0·1 cc. in each tube.
Emulsion of 24 hours agar culture of *B. typhosus*, 0·5 cc.
N/10 H_2SO_4, N/10 $H\bar{A}$ and N/10 $H_2\bar{T}$ or equivalent added in amounts indicated
Contents of each tube made to 1 cc. with distilled water.
2 hours at 37° C. 24 hours at room temperature.

N/10 H_2SO_4 ...	0	·010	·012	·014	·016	·018	·020	·022	·024
	+++	+++	+++	+++	+++	+++	+++	+++	+++
N/10 $H\bar{A}$ ...		·016	·018	·020	·022	·024	·026	·028	·030
		+++	+++	+++	+++	+++	+++	+++	+++
N/10 $H_2\bar{T}$ ...		·16	·18	·20	·22	·24	·26	·28	·30
		+++	+++	+++	+++	+++	+++	+++	+++

N/10 H_2SO_4 ...	·026	·028	·030	·032	·034	·036	·038	·040
	++	+	+	–	–	–	–	–
N/10 $H\bar{A}$ ...	·032	·036	·038	·04	·06	·08	·10	
	+++	+++	+++	+++	++	+	–	
N/10 $H_2\bar{T}$ ...	·32	·34	·36	·38	·40			
	+++	+++	+++	+++	+++			

The nature of the acid used has a marked influence. Thus in Table II it is seen that sulphuric acid inhibited in a lower concentration than did acetic acid, and tartaric acid had a feeble inhibitory effect. Although tartaric acid, in the dilutions used, did not inhibit the agglutination as observed at the end of twenty-four hours there was a considerable slowing of the reaction, and at the end of four hours there was no agglutination in the tubes containing more than 0·3 cc. N/10 tartaric acid.

This result is analogous to the results obtained by Chick and Martin in the agglutination of denaturated serum proteins.

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It is necessary to point out here that suspensions of denaturated proteins are agglutinated if acids be added in certain concentrations. If more acid be added the agglutinated proteins are again dispersed.

Chick and Martin found that the weakest acid—butyric acid in a particular experiment—gave a wider range within which the denaturated protein was agglutinated, than the stronger acids—acetic and hydrochloric. When the acids reached a certain concentration the protein was again dispersed, and a much greater equivalent amount of the weaker acid was needed than of the stronger to cause dispersion. These results, they maintain, are due to the differences in the H-ion concentration of equivalent dilutions of the acids.

(*c*) *Effects of relative proportions of acids or alkali and serum.*

Table III shows that in order to inhibit agglutination, the acid or alkali and the serum must be present in certain relative proportions. The greater the quantity of serum present, the greater is the amount of acid or alkali required to inhibit agglutination.

TABLE III.

Typhoid *v.* goat serum 0·1 cc., 0·01 cc., 0·001 cc. respectively in each tube in the three series.

0·5 cc. emulsion of *B. typhosus* in normal saline.

N/100 H_2SO_4 in amounts indicated.

Contents of each tube made up to 1 cc. with distilled water. Results after 24 hours at room temperature.

N/100 H_2SO_4 ...	0	0·08	0·10	0·12	0·14	0·16	0·18	0·20	0·22	0·24
0·1 cc. serum ...	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0·01 cc. „ ...	+++	+++	+++	+++	+++	+++	+++	++	++	+
0·001 cc. „ ...	+++	+++	+++	++	++	++	+	+	+	+
0 ...	−	−	−	−	−	−	−	−	−	−

Thus in the experiment detailed in Table III it was found that using 0·1 cc. of agglutinating serum, 0·24 cc. of N/100 sulphuric acid was insufficient to cause any inhibition, while with 0·001 cc. of serum there was almost complete inhibition with 0·18 cc. N/100 sulphuric acid. This may in part be due to adsorption of acid by serum proteins other than those concerned in the agglutination of the bacteria, but another explanation will be given later.

(*d*) *Effect of absolute concentration of acid or alkali.*

If serum be mixed with acids or alkalis and a series of dilutions of the mixture made and added to a suspension of homologous bacteria, a dilution will be reached where there is complete agglutination in spite of the presence of acids or alkali, provided that not too much acid or alkali has been added (see Table IV). From this it is evident that a certain absolute concentration of acid is necessary to produce inhibition.

TABLE IV.

Rabbit *v.* typhoid serum 1 : 10 in saline solution mixed with an equal volume of N/10 H_2SO_4 and following dilutions made with normal saline solution, the dilution being reckoned in terms of whole serum present in final mixture of serum, acid and bacterial emulsion.

Emulsion of *B. typhosus* in normal saline solution 0·5 cc. in each tube.

Contents of all tubes made to 1 cc. with saline. 3 hours incubator.

Serum and acid	1 : 100	1 : 200	1 : 400	1 : 600	1 : 800	1 : 1600	1 : 3200	1 : 6400
	−	−	−	−	+++	+++	++	−

This point is brought out also in another experiment in which different quantities of acid were added to a mixture of bacteria and a series of dilutions of specific immune serum (see Table V). It is seen that 0·4 cc. of N/100 sulphuric acid almost completely inhibited agglutination by 1/100 serum, while 0·04 cc. had no inhibitory action whatever.

TABLE V.

Rabbit *v.* typhoid serum in dilutions indicated.

Emulsion of *B. typhosus* in normal saline solution 0·5 cc. in each tube.

Amounts of N/100 H_2SO_4 as indicated.

Contents of each tube made up to 1 cc. with distilled water.

2 hours at 37° C. 24 hours room temperature.

Serum.

		1 : 100	1 : 200	1 : 400	1 : 800	1 : 1600	1 : 3200	1 : 6400	0
N/100 H_2SO_4	0·04 cc.	+++	+++	+++	+++	+++	++	+	−
	0·2 cc.	+++	++	+	+	+	+	+	−
	0·4 cc.	+	+	+	+	+	−	−	−
No acid	...	+++	+++	+++	+++	+++	++	+	−

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(e) *Time factor in acid or alkali inhibition.*

The interval of time elapsing between the addition of the acid or alkali and the mixing of the bacteria and serum is of importance. If the acid or alkali be added at the time the mixture of serum and bacteria is made, much less is required to inhibit the agglutination than if either be added some time later, and within certain limits the longer the time interval the greater is the amount of acid or alkali required. It will be seen in Table VI that if the amount of acid or alkali added be kept constant, inhibition is observed in the presence of greater concentrations of serum, if the addition be made at the time of mixing than if it be made later.

TABLE VI.

Rabbit *v.* typhoid serum in dilutions indicated.

Emulsion of 24 hours agar culture of *B. typhosus* in normal saline solution, 0·5 cc. in each tube.

0·1 cc. N/10 KOH added to each tube at stated intervals.

Contents of each tube made up to 1 cc. with normal saline solution.

3 hours at 37° C. after last addition. 24 hours at room temperature.

	Serum.								
Alkali added	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320	1 : 640	1 : 1280	1 : 2560
After 0 minutes	+++	+++	−	−	−	−	−	−	−
„ 30 „	+++	+++	+++	+++	+	−	−	−	−
„ 60 „	+++	+++	+++	+++	+++	+	−	−	−
„ 120 „	+++	+++	+++	+++	+++	+++	+++	+	−
Control ...	+++	+++	+++	+++	+++	+++	+++	+++	++

An explanation is probably to be found in the different states of aggregation of the agglutinated bacteria at different times after the commencement of the agglutination reaction. The precipitation of colloids from solution or suspension by electrolytes is apparently due to the adsorption by the particles of that ion of the electrolyte which bears an electric charge opposite in sign to their own. Particles bearing charges of the same sign repel one another. The adsorption of ions of opposite sign neutralises the charge carried by the particles and they are no longer mutually repelled, but come together and form aggregates which fall to the bottom of the containing vessel, the rate of fall depending on the size of the aggregates. If the charge given to the particles by the adsorbed ions be greater in amount than is necessary to neutralise the charge already borne by them, they will take on the opposite charge to that which they originally bore and will again repel one another and hence be dispersed.

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The aggregates of agglutinated bacteria can readily be observed to become larger and more coherent with the lapse of time, the rate at which this occurs depending, among other things, on the amount of agglutinating serum which is present.

Chick and Martin (1912) point out that the dispersion of particles of denaturated proteins by small amounts of acid or alkali, is due to the electric charge given to the particles. The particles of a loose aggregate can more readily take on this charge and be dispersed by it than the particles of a coherent mass, for in the latter case the aggregate is probably charged as a whole and any disruptive action is limited to the superficial particles and even here operates at great mechanical disadvantage.

The effect of acid or alkali in preventing agglutination may be explained in a similar way; the more coherent the aggregate of bacteria is, the smaller is the surface on which the charge can act. It follows, therefore, that the greater the degree of cohesion of the agglutinated bacteria, the greater is the charge necessary to disagglutinate them and hence the greater the amount of acid or alkali required. With a small amount of agglutinating serum the rate at which cohesion takes place is lower than with a large amount of agglutinating serum.

TABLE VII.

Typhoid bacilli from agar culture were agglutinated with (*A*) 1–10, (*B*) 1–100, (*C*) 1–1000 typhoid goat serum and after 4 hours were centrifuged and washed twice with distilled water then re-emulsified in distilled water. In each tube was placed 0·5 cc. of the respective emulsions, the indicated amount of N/100 H_2SO_4 or equivalent and distilled water to make the contents of each tube up to 1 cc.

N/100 H_2SO_4	·002	·004	·006	·008	·010	·012	·014	·016	·018
A 1– 10 ...	++	++	++	++	+++	+++	+++	+++	+++
B 1– 100 ...	–	–	–	–	–	+	++	+++	+++
C 1–1000 ...	–	–	–	–	–	–	–	–	–

N/100 H_2SO_4	·020	·04	·06	·08	·10	·12	·14	·16	0
A 1– 10 ...	+	+	+	+	+	–	–	–	–
B 1– 100 ...	+++	++	+	+	+	–	–	–	–
C 1–1000 ...	+	+++	+++	+++	++	+	+	+	–

Effects of acids on washed agglutinated bacteria.

If bacteria be agglutinated by specific sera, then washed free from serum and salts and emulsified in distilled water, they remain dispersed for a considerable length of time without re-agglutination taking place. The addition of low concentrations of acids to such dispersions may, however, cause re-agglutination.

The amount of acid required to re-agglutinate the bacteria depends upon the amount of agglutinating serum originally used to "sensitise" the bacteria. Numerous observers have shown that unsensitised bacteria may be agglutinated by certain concentrations of acids. The strain of *B. typhosus* used in these experiments showed partial agglutination between the limits of 0·06 cc. and 0·18 cc. of N/100 sulphuric acid when unsensitised. It is seen in the experiment detailed in Table VII that when the bacteria were sensitised with 1 : 10 serum 0·01 cc. to 0·018 cc. of N/100 sulphuric acid caused re-agglutination of the washed bacteria, while when they were sensitised with 1 : 1000 serum 0·04 cc. to 0·08 cc. of N/100 acid were required to cause re-agglutination. It is evident, then, that with the smaller amount of serummore acid is required than with the large.

Analogous results were obtained by Porges (1906), using sodium chloride in place of acid. Thus serum diluted 1 : 10 gave agglutination in the presence of 0·0002N sodium chloride, while serum 1 : 2000 required 0·02N sodium chloride for agglutination.

Tullock (1914) found that emulsions of *B. suipestifer*, with 1 : 500 antiparatyphosus B serum required the presence of N/80 sodium chloride to be agglutinated while *B. paratyphosus* B was agglutinated with the same dilution of serum in the presence of N/400 sodium chloride. This is evidently due to the presence of a smaller amount of group agglutinin which agglutinates *B. suipestifer*, than of the specific agglutinin.

TABLE VIII.

Calf serum diluted 1 : 10, 1 : 50 and 1 : 100 respectively in distilled water boiled for a few seconds. 0·5 cc. serum dilution in each tube. Amount of N/100 H_2SO_4 or equivalent as indicated. Contents of all tubes made up to 1 cc. with distilled water.

N/100 H_2SO_4	...	0	·02	·04	·06	·08	·10	·12	·14	·16
Serum 1 : 10	...	–	–	–	–	c	+++	+++	+++	+++
N/100 H_2SO_4	...	0	·002	·004	·006	·008	·010	·012	·014	·016
Serum 1 : 50	...	–	–	–	–	–	–	–	–	–
N/100 H_2SO_4	...	0	·002	·004	·006	·008	·010	·012	·014	·016
Serum 1 : 100	...	–	–	–	–	–	sc	c	+++	c
N/100 H_2SO_4	...	·18	·20	·22	·24	·26	·28	·30	·32	·34
Serum 1 : 10	...	+	sc	sc	–	–	–	–	–	–
N/100 H_2SO_4	...	·018	·02	·04	·06	·08	·10	·12	·14	·16
Serum 1 : 50	...	sc	c	+++	–	–	–	–	–	–
N/100 H_2SO_4	...	·018	·02	·04	·06	·08	·10	·12	·14	·16
Serum 1 : 100	...	c	sc	–	–	–	–	–	–	–

+++ = Large precipitate, fluid clear.
\+ = Slight precipitate, fluid cloudy.
c = Cloudy. No precipitate.
sc = Slightly cloudy.
– = Clear. No precipitate.

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As an explanation of these observations the following is suggested:

The agglutination of bacteria is dependent on two opposing tendencies: (1) the tendency of the bacteria to remain dispersed; (2) the tendency of the agglutinin to cause aggregation of the bacteria.

The aggregation of bacteria "sensitised" by agglutinating serum is probably due to the neutralisation of the electric charge carried by the bacterium-serum protein complexes, just as the aggregation of particles of denaturated protein is occasioned by neutralisation of the charges originally carried by them.

When a high dilution of agglutinating serum is used to agglutinate bacteria a smaller amount of serum protein is fixed in relationship to the bacteria than when a lower dilution is used. If "sensitisation" of the bacteria be accompanied by a partial electric discharge following the formation of the bacterium-serum protein complex, it follows that a larger amount of acid will be necessary to produce aggregation when there is little protein fixed than when there is much. For, in the former case, the force exerted to prevent aggregation will have been neutralised by the serum protein fixed to a less degree than in the latter.

The amount of acid required to agglutinate denaturated serum protein depends upon the amount of protein present as shown in Table VIII. It is seen that serum diluted 1 : 10 with distilled water and boiled required the addition of 0·1 to 0·16 cc. of N/100 sulphuric acid to cause agglutination while serum diluted 1 : 100 required only 0·014 cc. of N/100 acid.

TABLE IX.

Precipitate from the interaction of calf serum and goat *v.* calf serum was washed twice with distilled water and re-emulsified in distilled water; 0·5 cc. emulsion in each tube and amounts of N/1000 H_2SO_4 as indicated. Contents of all tubes made to 1 cc. with distilled water.

N/1000 H_2SO_4 ...	0	·02	·04	·06	·08	·10	·12	·14	·16
	−	+++	+++	++	+	−	−	−	−

The precipitate in a precipitin reaction if washed free from serum and salts and emulsified in distilled water, acts in a similar fashion to agglutinated bacteria in the presence of acids, being precipitated by small concentrations of acids and redispersed by greater concentrations (see Table IX).

Heated serum euglobulin acts also in a similar manner. Euglobulin was prepared from calf serum by diluting 1 : 10 in distilled water and saturating with carbon dioxide. After half an hour the

precipitate of euglobulin, which was formed, was separated, washed with and emulsified in distilled water forming a cloudy suspension. One half of the suspension was boiled for a few seconds after having been thoroughly shaken in order to disperse the particles completely. The heated euglobulin suspension has been kept for weeks without showing any sign of precipitation.

TABLE X.

In each tube 0·5 cc. of either heated or unheated euglobulin suspension in distilled water and N/100 H_2SO_4 or equivalent in amounts indicated.

Contents of all tubes made up to 1 cc. with distilled water.

N/100 H_2SO_4 ...	·002	·004	·006	·008	·010	·012	·014
Unheated euglobulin	T	T	T	T	+++	+++	+++
Heated euglobulin ...	T	T	T	+	++	+++	+++
N/100 H_2SO_4 ...	·016	·018	·020	·04	·06	·08	
Unheated euglobulin	+++	+++	c	c	c	c	
Heated euglobulin ...	+	+	T	T	T	T	

T = turbid. c = clear.
+++ = large precipitate.
++ = large precipitate with marked turbidity.
+ = small precipitate.

It will be seen in Table X that the addition of more acid than was required to agglutinate the unheated euglobulin caused the suspension to clear completely. In the case of the heated euglobulin, on the other hand, there was a zone in which agglutination occurred, the tubes containing a smaller amount of acid than 0·008 cc. or a greater amount than 0·02 cc. being equally turbid. The resemblance in the appearance of the contents of the tubes in the heated euglobulin series to those of an analogous series using washed agglutinated bacteria was remarkably close.

TABLE XI.

In each tube was placed 0·5 cc. of the heated serum + bacteria (S + B) mixture, of the heated serum (S), or of the heated bacterial suspension (B).

N/100 H_2SO_4 was added in the amounts indicated and the contents of each tube made up to 1 cc. with distilled water.

N/100 H_2SO_4 ...	0	·10	·12	·14	·16	·18	·20	·22	·24	·26
S ...	–	–	–	cloudy	++	+++	+++	+++	+++	+++
S + B ...	–	–	–	–	++	+++	++	++	+	+
B ...	–	–	–	–	–	–	–	–	–	–

+++ = complete agglutination or large precipitate.
++ = large amount of agglutination or precipitate not so large.
+ = slight precipitate.
– = no agglutination or no precipitate

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The precipitation of denaturated serum proteins by acids may occasion the aggregation of bacteria suspended in the mixture. An emulsion of *B. dysenteriae* was made in calf serum diluted 1 : 10 with distilled water and after standing for five minutes, the emulsion was boiled for a few seconds. The same serum diluted 1 : 10 in distilled water and an emulsion of *B. dysenteriae* in distilled water were also boiled for a few seconds. The *B. dysenteriae* was not agglutinated by the unheated calf serum in any dilution.

It will be seen in Table XI that there was only one tube in which the bacteria were completely agglutinated so that the formation of a large precipitate of denaturated protein does not necessarily result in the carrying down of the bacteria. This probably has to do with the rapidity of aggregation of the particles of denaturated protein. If the particles be aggregated rapidly, as they were in the tubes with the larger amounts of acid, the bacteria probably have less chance of becoming entangled in the precipitate, than if they be aggregated more slowly.

Attention may be drawn in this connection to the observations of H. R. Dean (1911) on the deviation of complement in precipitin reactions. He found that if a precipitate be formed rapidly, there was little, if any, deviation of complement while a very slowly forming precipitate deviated a considerable amount of complement.

A consideration of the experiments detailed in Tables VII–XI show that there is an undoubted analogy in the results obtained. Bacteria "sensitised" by specific agglutinating serum, precipitate from a precipitin reaction, and denaturated euglobulin were all agglutinated by low concentrations of acids and dispersed by higher concentrations and all showed a range of optimum acidity for agglutination. The similarity of the results would seem to justify the assumption that the reactions are expressions of the same process and strengthens the conception that the precipitin reaction is due, in the main, to the precipitation of altered serum globulin and that agglutination of bacteria in an agglutination reaction follows as a sequence to the precipitation of altered serum globulin.

Effects of heat on the properties of agglutinating serum.

A considerable amount of work has been done on the effect of high temperatures on agglutinating serum.

It is found that if agglutinating sera be heated for half an hour at certain temperatures—varying according to the serum employed but

usually in the vicinity of 60°–70° C.—the serum may no longer produce agglutination in any dilution. If the serum be heated for half an hour, at a higher temperature—usually about 75° C.—the serum may become, not only inactive, but also inhibitory, that is the addition of a sufficient amount to a mixture of unheated serum and bacteria, will inhibit the agglutination. Heating at still higher temperatures will destroy this inhibitory effect, while the serum still remains inactive.

TABLE XII.

Goat *v.* typhoid serum diluted 1 : 10 with distilled water.

Parts of the dilution were heated at 75° C. in a water bath for 10 minutes, 20 minutes and 30 minutes respectively.

Dilutions of serum as indicated, the dilutions being given in terms of undiluted serum.

Emulsions of 24 hours agar culture of *B. typhosus* 0·5 cc. in each tube.

Contents of each tube made up to 1 cc. with saline.

4 hours at 37° C. Room temperature 18 hours.

	·01	·005	·0025	·00125	·000625	·000312	·000156	·000078
Heated 0 minutes	+++	+++	+++	+++	++	+	–	–
,, 10 ,,	+	++	++	+	–	–	–	–
,, 20 ,,	–	+	+	–	–	–	–	–
,, 30 ,,	–	–	–	–	–	–	–	–

If different lots of a dilution of serum be heated for different times, another phenomenon may be observed—the production of zones of inactivation. It is seen in Table XII that in this particular experiment heating for twenty minutes did not inactivate the serum completely, but that complete inactivation was observed in low and high dilutions, 1/100 and 1/800, but incomplete in a middle zone. In another experiment where 1 : 5 goat *v.* typhoid serum was heated at 77° C. for twenty minutes, there was complete agglutination with 0·0025 cc. of serum, but only a trace with 0·02 cc.

It is probable that in these cases, the inactivation is due to the presence of inhibitory substance in sufficient amounts in the high concentrations to produce inhibition.

Serum heated but not completely inactivated may, in certain concentrations, inhibit the agglutination by unheated serum (see Table XIII).

Heated immune sera vary considerably in their ability to produce inhibition. Some heated sera will inhibit the agglutination by an equal concentration of unheated serum, provided the dilution is not great, while other heated sera must be added in much greater amounts to cause inhibition. Thus, in the experiment detailed in Table XIII, 0·02 cc. of heated serum completely inhibited the agglutination by 0·00125 cc. of unheated serum, but not of 0·0025 cc.

TABLE XIII.

Rabbit *v.* typhoid serum 1 : 5 in distilled water heated at 75° C. in a water bath for half an hour.

Dilutions of serum in terms of original serum.

0·5 cc. emulsion of *B. typhosus* in each tube.

Contents made up to 1 cc. with normal saline solution.

3 hours at 37° C. 18 hours at room temperature.

	0·08	·01	·005	·0025	·00125	·000625	·000312	·000156	·000078	·000039
Unheated serum	.	+++	+++	+++	+++	+++	+++	++	+	–
Ditto + 0·02 cc. heated serum	.	+++	++	+	–	–	–	–	–	–
Ditto + 0·08 cc. heated serum	.	–	–	–	–	–	–	–	–	–
Heated serum	–	+	++	++	++	++	++	++	+	–

Eisenberg (1906) showed that high concentrations of heated sera inhibited agglutinations to a greater extent than low concentrations. Thus he found that heated serum 1/100 did not completely inhibit unheated serum 1/500, while heated serum 1/10 almost completely inhibited unheated serum 1/10.

There is a close resemblance between the effects of heated serum and the effects of acids and alkalis in preventing agglutination.

TABLE XIV.

Goat *v.* typhoid serum in dilution 1 : 5 in distilled water heated at 75° C. in water bath for half hour 0·4 cc. heated serum dilution added to each tube of series 1, 2 and 3 after intervals noted and 0·4 cc. distilled water to series 4.

Dilutions of unheated serum as indicated.

0·5 cc. emulsion of 24 hours agar culture of *B. typhosus* in normal saline solution in each tube.

	·05	·025	·0125	·00625	·00312	·00156	·00078	·00039	·00019
Heated serum added at once	+++	+++	+++	+++	+	–	–	–	–
After 10 minutes	+++	+++	+++	+++	+++	+++	+	–	–
„ 60 „	+++	+++	+++	+++	+++	+++	+++	+	–
No heated serum	+++	+++	+++	+++	+++	+++	+++	+++	++

Thus in both cases the zone phenomenon may be observed, and in both there is more inhibition with high concentrations than with low. (Compare Tables V and XIII.)

As with inhibition with acids and alkalis, there is less inhibition the later the heated serum is added to the mixture of unheated serum and bacteria. (Compare Tables XIV and VI.)

If bacteria be agglutinated by specific sera and the supernatant fluid removed and replaced by heated, inhibitory serum, a certain

amount of dispersion may take place or the bacteria may even be completely dispersed. Bacteria agglutinated by the group agglutinin of heterologous sera, are somewhat more readily dispersed by heated specific sera (see Table XV). This is probably due to the fact that there is much less group agglutinin, than specific agglutinin, in a specific agglutinating serum.

TABLE XV.

Rabbit *v.* typhoid serum and rabbit *v.* paratyphoid A serum. Emulsion of *B. typhosus* in saline. After 24 hours supernatant fluid removed and rabbit *v.* typhoid or rabbit *v.* paratyphoid A serum 1 : 5 in distilled water heated at 75° C. for half hour added. Contents made up to 1 cc.

Dilutions of serum in terms of original serum.

Typhoid Serum	Para. A Serum	*B. Typh.* Emulsion	After 24 hours		Typhoid Serum (75)	Para. A Serum (75)	
0·05 cc.	—	0·5 cc.	+++	Supernatant fluid removed and added	0·1	—	++
0·05 cc.	—	0·5 cc.	+++		—	0·1	++
—	0·05 cc.	0·5 cc.	+++		0·1	—	+
—	0·05 cc.	0·5 cc.	+++		—	0·1	++

Different observers have obtained analogous results with precipitin anti-sera. Precipitin anti-sera are inactivated by heat and heating at high temperatures produces inhibitory properties.

The zone phenomena of heated agglutinating sera find a parallel in heated precipitin sera.

The relative inhibitory value of different heated precipitin sera varies, just as does that of heated agglutinating sera.

The time factor has the same influence with heated precipitin anti-sera as with heated agglutinating sera. Finally, as Welsh and Chapman (1909) have shown, heated precipitin anti-sera may dissolve homologous precipitate and, to some extent, heterologous precipitate also.

The interpretation of these phenomena of inactivation and inhibition is, at present, impossible. The resemblance between the effects of inactivation and inhibitory properties in heated agglutinating sera and the effects of acids and alkalis is noteworthy. The inhibitory action of heated serum is not, however, due to the production of acid or alkali in the serum, at any rate in amounts which can be demonstrated by the determination of the H-ion concentration by Sörensen's indicator method. A heated, inhibitory goat *v.* typhoid serum in dilution 1 : 10 in distilled water showed the same H-ion concentration as did the unheated serum in the same dilution.

The adherents of the Ehrlich school consider that the inhibitory action is due to the production of pro-agglutinoids and pro-precipitoids, which have a greater affinity for the antigen than have agglutinins or precipitins. The observation of Welsh and Chapman (1909) that the addition of a considerable excess of antigen did not bring the inhibition in a heated precipitin serum to an end, renders this hypothesis untenable in the precipitin reaction and probably in the agglutination reaction also.

The nature of the change in the heated serum has not been determined, but in the precipitin reaction the amount of inhibitory action seems to depend upon the amount of "precipitable substance" present in the serum and to be intimately connected with it.

Welsh and Chapman consider the fact that heating a precipitin serum to a certain temperature renders it inactive and that heating it to a higher temperature renders it inhibitory is "corroborative evidence of the conclusions, that inactivation is not due to the development of an inhibitory substance in the antiserum and that the onset of inactivation does not determine the onset of inhibition." They seem to have quite overlooked the phenomenon of "zones of inactivation."

Dreyer (1904) has shown in the case of agglutinating sera that if two sera be heated they may both show a large zone of inhibited agglutination, but in one case the titre of the serum may be much reduced, while in the other case, the serum may not be appreciably weakened.

It seems, however, much more likely that the inactivation produced by heating serum at temperatures of 60°–72° C., observed in the low dilutions is due to the formation of an inhibitory substance which, when considerably diluted, cannot produce any effect, just as was seen in the case of acids and alkalis.

The inactivation produced by heating serum to 80° C. or higher is, in all probability, due to the coagulation of the serum proteins and the removal of the agglutinins or precipitins from the solution.

Chick and Martin (1910) have pointed out that the heat coagulation of proteins—or denaturation—does not take place at any definite temperature, but is merely accelerated by raising the temperature—thus with an egg albumin solution—it took 617 minutes to reduce the amount of protein retained in solution from 9 mgm. to 3 mgm. per cubic centimetre 69° C., while it took only 7·2 minutes at 76·3° C.

It is suggestive that the inactivation and production of inhibitory properties in immune serum increase—up to a maximum—with increasing times of heating, and with increase of heat, provided the temperature is not too high. It seems possible that these effects are

due to the denaturation of a part of the serum proteins, probably those fractions which are intimately connected with the agglutinins and precipitins.

Conclusions.

1. The agglutination and precipitin reactions are probably essentially the same in nature.

2. The agglutination of bacteria by specific sera, is probably due to the formation of altered serum protein, in and around the bacteria and the subsequent flocculation, by electrolytes, of this altered protein and the bacteria.

3. This altered protein is probably altered serum globulin, and possibly other altered serum proteins.

4. The phenomenon of inhibition, exhibited by heated agglutinating serum, resembles closely the inhibition of agglutination by acids and alkalis.

5. The inactivation of agglutinating serum by heating at temperatures between about 60° and 72° C., and the production of "zones of inactivation," are probably due to the development of inhibitory substances and not to destruction of the agglutinin.

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