XXXVII. THE MECHANISM OF AGGLUTINATION OF BACTERIA BY SPECIFIC SERA.

By WILLIAM JOHN TULLOCH.

*From the Bacteriological Laboratory of the University of Durham College
of Medicine, Newcastle-upon-Tyne.*

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Flocculation of bacteria and other cells by specific antisera, being, if not the simplest, at least the most easily investigated of the "in vitro" phenomena of immunity, offers a suitable field for the study of the mechanism of serum tests in general, for if we knew the factors that control agglutination we might be able to correlate this phenomenon with others—e.g. lysis—which, while apparently very different therefrom, may in reality be closely allied thereto. The immediate purpose of the investigation was, then, to enquire into the factors determining flocculation of sensitised bacteria; while it was hoped that from a study of these an indication for the explanation of biological lysis and other laboratory serum tests on a basis of observed physiological fact might be obtained.

The experiments to be described in the present communication were carried out with a view to the corroboration and amplification of a statement made by Bordet [1899]: "That agglutination depends upon the presence of an electrolyte in the fluid used for suspending the organisms." This author showed that bacteria that have been exposed to the action of specific antiserum, then washed, and suspended in distilled water, form an homogeneous and stable suspension. The stability of this suspension of "sensitised" organisms depends upon the absence of electrolytes from the fluid as is shown by the fact that the addition of a small quantity of NaCl results in complete agglutination of the suspended organisms.

This experiment might be interpreted as indicating that the process of agglutination by antisera is divisible into two phases:

(a) A phase in which the antibody of the serum becomes united with its antigen—the organism;

(b) A phase in which the united "antibody-antigen couple" is flocculated by the electrolyte of the suspending fluid.

The second phase of the phenomenon is purely physical in character and is comparable with the sedimentation of sand at river estuaries and with the aggregation of particles of "denaturated" protein by the addition of small quantities of electrolytes. It is with the second phase of the reaction that the experiments to be described are mainly concerned.

Before the publication of Bordet's observation [1899] there had been a number of explanations offered of the nature of the reaction of specific agglutination. These were all founded upon chemical bases and failed to explain satisfactorily the clumping of organisms by specific antisera. Thus Gruber and Durham [1896] considered that the serum produced a chemical (?) change in the outer layers of the organismal protoplasm causing them to become sticky. This view of the phenomenon while offering adequate explanation of the mutual adhesion of sensitised bacteria or cells, when they had come into contact with one another, failed altogether to explain why they *should come together to form clumps*.

The next advance made in the investigation and explanation of specific agglutination is due to Krause [1897], who showed that the clear fluid obtained by filtration of an old broth culture of an organism produced a precipitate when mixed with an homologous antiserum. Basing their observations on this fact, Nicolle [1898] and Paltauf [1897] advanced the following hypotheses. The former considered that the phenomenon was really explicable by the theory advanced by Gruber and Durham, but he claimed that that moiety of the organismal protoplasm which was susceptible to the action of the agglutinating serum—"agglutinable substance"—was resident principally in the external layers of the organism and might in old cultures diffuse into the medium. Its precipitation in the organisms themselves led to agglutination while its flocculation from the culture filtrate led to the precipitum observed by Krause. It will be seen that Nicolle's theory involves the acceptance of Gruber's hypothesis and only differs therefrom in correlating the Krause phenomenon with agglutination. Paltauf considers that in all cultures a certain amount of agglutinable substance diffuses into the medium and is precipitated by the addition of antiserum, so forming a coagulum which entangles the suspended bacteria and subsequently contracting leads to the formation of agglutinated masses.

This view of Paltauf's is ingenious, but it presupposes that a coagulum is always formed in the suspending fluid and that this coagulum *is not*

discontinuous. As there is no evidence that these suppositions are valid, the explanation offered must be considered as inadequate. His hypothesis, however, seems to me to be of considerable import in that it draws attention, indirectly at least, to the physical state of the interacting bodies as an important factor in the phenomenon of agglutination.

From the experiments to be described it will be noted that a striking analogy may be drawn between the behaviour of sensitised organisms and that of suspensions of denaturated protein in presence of electrolytes. Furthermore, unsensitised bacteria behave in respect of electrolytes as does fresh protein, and Liefman [1913] has shown that in order to produce flocculation of unsensitised organisms a concentration of 70–80 % $(\text{NH}_4)_2\text{SO}_4$ is required. With different organisms different concentrations of the salt appear to give optimum results, therein exhibiting a striking resemblance to the fractional salting out of proteins from solution by similar reagents. Agglutination of sensitised bacteria on the contrary occurs in presence of, and is conditioned by low concentrations of, salts, e.g. physiological saline, and has, I think, its analogue in the aggregation of denaturated protein particles by low concentrations of salts. This subject of the aggregation of denaturated protein by the addition of salts has been fully investigated by Chick and Martin [1912], who show that flocculation under these circumstances depends upon the neutralisation of the surface electric charge carried, according to the Lippman-Helmholtz hypothesis, by particles or molecules in suspension.

If the hypothesis, then, “that aggregation of denaturated protein particles and agglutination of sensitised bacteria are similar phenomena” be correct, we should expect sensitised bacteria to exhibit certain of the features characteristic of denaturated protein. In respect of electrolytes, particles of denaturated protein recall, in certain respects, in their reactions those of rigid colloids; thus the charge carried by such colloids is largely determined by the reaction of the suspending fluid, and the valency of the ions of the electrolyte in which the colloid is suspended is an important factor in its precipitation. We should expect then, if our hypothesis be correct, that the presence of hydrion and hydroxidion would markedly affect the process of agglutination and that in certain experimental conditions the valency of the kation of the electrolyte would determine the aggregation of the organisms while in other circumstances the clumping would be conditioned by the valency of the anion.

EXPERIMENTS TO SHOW THAT VARIATION OF THE INTRINSIC PRESSURE
AND VISCOSITY OF THE SUSPENDING FLUID HAS NO MARKED INFLUENCE
ON THE REACTION.

As the disposition of particles suspended in a fluid is largely determined by the electric charge carried by the particles and by the intrinsic pressure of the suspending fluid, one can alter the disposition of the particles by interfering with either of these factors. These are not infrequently interdependent, but without altering the intrinsic pressure of a fluid we may bring about flocculation of particles suspended in it by neutralisation of the surface charge carried by the particles, and I conceive of agglutination as being dependent on this mechanism. It seemed possible, however, that flocculation of organisms by antisera might depend upon alteration of the intrinsic pressure of the suspending fluid, and with a view to elucidating this point, agglutination reactions were carried out in presence of 0.9 % NaCl, 0.9 % NaCl dissolved in 10 % alcohol, and 0.9 % NaCl dissolved in 1 % gelatin.

Examination of these fluids by means of Traube's stalagmometer showed that for a given volume of the fluids at 37°—the temperature at which the reactions were carried out—

0.9 % NaCl equalled	58.5 " standard " drops,
0.9 % NaCl in 10 % alcohol =	89.5 drops,
0.9 % NaCl in 1 % gelatin =	65.2 drops,

thus indicating a very considerable difference in the intrinsic pressure of the solution in alcohol as compared with the others.

Employing the viscosimeter and working at the same temperature, it was found that for a given volume to pass through the tube

0.9 % NaCl	required 2 min. 15 secs.,
0.9 % NaCl in 10 % alcohol	required 3 min. 20 secs.,
0.9 % NaCl in 1 % gelatin	required 3 min. 15 secs.,

thus showing a marked increase in the internal friction in both the alcoholic solution and the gelatin solution.

The following agglutination reaction was carried out, using the above fluids for diluting the serum and for suspending the organisms. Throughout the experiments the organism used was *B. paratyphosus* "b."

TABLE I. 2 hours at 37°.

Fluid employed	Dilutions of agglutinating serum						
	1/100	1/500	1/1000	1/2000	1/4000	1/8000	1/16000
0.9 % NaCl	+	+	+	+	+	+	-
„ in 10 % alcohol..	+	+	+	+	+	-	-
„ in 1 % gelatin ..	+	+	+	+	+	+	-

Note. In this and the following experiments + indicates a positive result.

From this it is seen that notwithstanding considerable variation in the intrinsic pressure and internal friction of the fluids in which the reaction was carried out, the result is not markedly different in the case of any of the fluids.

Flocculation would appear then to be due to a direct interaction of “antibody-antigen” complex with electrolyte, and, within the limits of the experiment at least, to be independent of the intrinsic pressure and viscosity of the suspending fluid. This agrees with the findings of Gengou [1908], who states that the flocking out of red corpuscles from suspension by the addition of colloids is due “fundamentally to a direct interaction of the two bodies,” *i.e.* colloid and cell.

It was shown by the same author that alteration of the physical state of the interacting bodies leads on the contrary to very different results. Thus if a suspension of BaSO_4 be added to fresh serum, the BaSO_4 is held in suspension, but its addition to the same serum previously heated to 65° results in sedimentation.

According to Gengou's views, when the BaSO_4 is added to fresh serum it remains dispersed because, adhering to the colloidal particles of the serum which show no tendency to spontaneous flocculation, the particles of BaSO_4 are kept apart from one another. Added to heated serum or to red blood cells the BaSO_4 does rapidly sediment, for both these colloids tend to flocculate of their own accord and naturally do so more rapidly when they have adsorbed the heavy BaSO_4 .

I quote these experiments in order to emphasise the important influence that the physical state of the reacting bodies has upon the phenomena of immunity reactions and to call attention to the points that require special consideration in the investigation of these, particularly in respect of agglutination.

INFLUENCE OF THE ELECTROLYTE UPON THE TITRE OF THE SERUM.

In view of the findings of the above experiments, I decided to proceed with the investigation of the reaction from the standpoint of its probable analogy with the aggregation of denaturated protein particles.

In the first series of experiments, agglutination tests were performed in presence of a variety of electrolytes in order to determine whether any of the electrolytes employed affected the titre of the agglutinating serum.

Technique. A 24 hours agar culture of the organism (*B. paratyphosus* "b") was washed off in distilled water and centrifuged, the supernatant fluid was pipetted off and to the deposit was added distilled water, the mixture well shaken and again centrifuged. This process was repeated twice and the deposit obtained after the last centrifuging was triturated and suspended in distilled water. To ensure the homogeneity of this suspension it was filtered through coarse filter paper.

This distilled water suspension was exposed to the action of varying dilutions of serum in presence of different electrolytes, the concentration of which was so arranged that with the addition of the distilled water suspension to each tube the electrolytes were equimolecular with 0.9 % NaCl. Employing this technique the following result was obtained :

TABLE II. 2 hours at 37°.

		Dilutions of Serum						
		1/100	1/500	1/1000	1/2000	1/4000	1/8000	1/16000
NaCl	..	+	+	+	+	+	+	-
NaF	..	+	+	+	+	+	+	-
Na ₂ SO ₄	..	+	+	+	+	+	+	-
K ₂ SO ₄	..	+	+	+	+	+	+	-
Na ₂ CO ₃	..	-	-	-	-	-	-	-
Na ₂ HPO ₄	..	+	+	+	+	+	+	-
BaCl ₂	..	+	+	+	+	+	+	-
Na ₃ Cit.	..	+	+	+	+	+	+	-
Aq. Dest.	..	+	-	-	-	-	-	-

Note. The positive result obtained with 1/100 dilution of the serum in water is probably due to the salts that the serum contains.

In the above reaction the concentrations of serum used were chosen with a view to showing whether any of the salts employed exercised a *marked* influence upon the titre of the serum.

From the above table the following points are made clear :

1. That for all the salts employed, in the above experimental conditions, the titre of the serum remains the same, provided that the solutions of the salts give an approximately neutral reaction.

2. That the clumping of sensitised organisms is independent of the presence of the Ca ion. If it were dependent on the presence of the Ca ion, agglutination would probably not occur in presence of fluoride.

I draw attention to this on account of the important function that Ca subserves in many vital phenomena.

3. That salts which hydrolyse to produce OH ions have not the property of agglutinating bacteria in presence of specific agglutinating serum.

This point is shown more strikingly in the following table in which are compared the actions of "acid" and "basic" carbonates and phosphates of sodium.

TABLE III. 2 hours at 37°.

		Dilutions of Serum						
		1/100	1/500	1/1000	1/2000	1/4000	1/8000	1/16000
NaCl	..	+	+	+	+	+	+	-
Na ₂ CO ₃	..	-	-	-	-	-	-	-
NaHCO ₃	..	+	+	+	+	+	-	-
Na ₃ PO ₄	..	-	-	-	-	-	-	-
Na ₂ HPO ₄		+	+	+	+	+	+	-

EXPERIMENTS ON THE INHIBITION OF AGGLUTINATION BY ALKALIS.

This inhibitory action of alkalis upon agglutination is of considerable interest in view of the influence that the reaction of the suspending fluid has upon the aggregation of particles of "denaturated" protein [Chick and Martin 1912; Hardy 1899], and the following series of tests was set up with a view further to elucidate the conditions that determined the inhibitory action of alkalis upon the flocculation of sensitised bacteria.

It might well be that the inhibition is essentially due to the same cause in each case and that this is probable is shown from the following results.

Technique. A 24 hours agar culture was washed off in saline and agglutinated at 22° in presence of 1/1000 agglutinating serum (titre—1/8000). The supernatant fluid was pipetted off and the material was washed twice with distilled water as in the previous series, then triturated, filtered and exposed to the action of varying dilutions of NaOH in presence of the following electrolytes each of which was, when all the reagents were added, equimolecular with 0.9 % NaCl.

TABLE IV. 2 hours at 37°.

	NaOH N/100.										
	No NaOH Control	1 cc.	0·9 cc.	0·8 cc.	0·7 cc.	0·6 cc.	0·5 cc.	0·4 cc.	0·3 cc.	0·2 cc.	0·1 cc.
NaCl	.. +	-	-	-	-	-	-	-	-	-	+
K ₂ SO ₄	.. +	-	-	-	-	-	-	-	-	-	+
KI	.. +	-	-	-	-	-	-	-	-	-	-
NaHCO ₃	.. +	+	+	+	+	+	+	+	+	+	+
Na ₂ HPO ₄	+	+	+	+	+	+	+	+	+	+	+

This shows that until the OH ion is present in the suspending fluid in a concentration equal to that in N/500 NaOH it fails to inhibit agglutination. This concentration is presumably not reached in the case of NaHCO₃ and Na₂HPO₄ in the above experimental conditions, for the addition of the NaOH to the solutions leads probably to an increase in the quantity of ionised Na₂CO₃ and Na₃PO₄ in the solutions of these "acid" salts, this being necessarily accompanied by a removal from the fluid of OH ions available for the inhibitory action.

As this experiment showed that salts having a "replaceable" H group in the anion behaved in a manner different from other salts in respect of this alkali inhibition, it appeared of interest to show that salts containing an "irreplaceable" H group in the anion did not exhibit the same peculiarity.

Technique. A 24 hours agar culture was washed off with distilled water, then washed and centrifuged twice. The deposit obtained was suspended in distilled water and added to tubes containing serum and electrolyte of such concentration that with the addition of the emulsion the following dilutions of serum were obtained, the electrolytes being equal in concentration to 0·9 % NaCl.

TABLE V. 2 hours at 37°.

	Dilutions of Serum						
	1/100	1/500	1/1000	1/2000	1/4000	1/8000	1/16000
NaCl	+	+	+	+	+	+	-
NaCl in N/100 NaOH	-	-	-	-	-	-	-
CH ₃ COONa	+	+	+	+	+	+	-
CH ₃ COONa	..	..	-	-	-	-	-
CCl ₃ COONa	..	..	+	+	+	+	-
CCl ₃ COONa	..	..	-	-	-	-	-
NaHCO ₃	+	+	+	+	+	+	-
NaHCO ₃	..	..	+	+	+	+	-

The inhibition of agglutination by the hydroxidion is probably akin to the prevention of aggregation of protein particles in presence of an alkali first noted by Hardy and interpreted by him as a phenomenon depending

upon disturbance of the electrostatic condition of the particles in suspension. For optimum aggregation the reaction must occur in a solution that is approximately neutral, for protein particles acquire a positive charge when suspended in acid fluids and a negative charge when suspended in alkaline fluids.

To obtain the best conditions for precipitation, this charge must be neutralised, *i.e.* the solution must be so adjusted that it is "isoelectric" with the particles.

It appears probable then that the agglutination of sensitised bacteria also depends on their being suspended in an "isoelectric" fluid.

In what way the alkali produces the inhibition of clumping is by no means clear, for it might obviously act upon the serum, the organism, the union of the serum and organism, or upon the actual flocculation.

From what has been said above, the last of these is seemingly the most rational explanation of the dispersion.

Paal and Voss [1904] suggest that the inhibition of coagulation (aggregation) of protein by hydroxidion depends upon the alkali acting upon the protein and producing a degree of hydrolysis that results in the production of a layer of protective colloid around each particle in suspension. If such a colloid is produced by the action of the OH ion on bacterial protoplasm, its properties disappear when the organisms exposed to the alkali are well washed in distilled water as is shown by the following experiment.

Technique. A 24 hours agar culture of organisms was washed off with N/100 NaOH and exposed to this reagent for 2 hours at 37°. The suspension was then centrifuged, the supernatant fluid pipetted off and replaced by distilled water. The mixture was well shaken and again centrifuged. This washing process was repeated twice and the organisms were then exposed to the following dilutions of an agglutinating serum.

TABLE VI. 2 hours at 37°. *Electrolyte used, 0.9 % NaCl.*

	Dilutions of Serum						
	1/100	1/500	1/1000	1/2000	1/4000	1/8000	1/16000
Untreated organisms	+	+	+	+	+	?	-
Treated as above	+	+	+	+	+	?	-

From this it is seen that organisms that have been exposed to the action of alkali and then washed react in the same way as do organisms that have not been so treated. This shows that the OH ion has no permanent effect upon the protoplasm of the bacteria.

Further, that the alkali has no permanent effect on the serum is shown in the following experiment.

Technique. Serum was diluted 1/50 in N/100 NaOH in 0.9 % NaCl. This mixture was placed in the incubator at 37° for 2 hours and was then diluted with 0.9 % NaCl to obtain the following dilutions of serum when the suspension of organisms—in saline—was added to the tubes. In this way both serum and alkali were being diluted in the series.

TABLE VII. 2 hours at 37°.

		Dilutions of Serum						
		1/100	1/500	1/1000	1/2000	1/4000	1/8000	16000
Serum alone	..	+	+	+	+	+	?	-
Serum plus alkali		-	-	+	+	+	-	-

As is seen, the agglutination is negative in the tubes containing the higher concentrations of serum plus the higher concentration of alkali, the results become positive with dilution of the reagents. It is apparent then that the alkali has no permanent effect on the serum.

The inhibition of clumping in alkaline suspension is due then to the presence of the OH ion and does not depend upon any permanent effect that the alkali has upon either the organisms or the serum.

The probability is that the inhibition is dependent upon alteration of the electrostatic condition of the suspended organisms, and is therefore the exact counterpart of the dispersion of denaturated protein particles exposed to similar reagents. It is of interest to note in this connection that Chick and Martin [1912] failed to show evidence of hydrolysis of pure protein heated to 100° for 2 hours in presence of sufficient alkali to prevent coagulation¹.

¹ This influence of the OH ion may possibly account for certain cases of "negative phase" that occur in high concentrations of an agglutinating serum. Thus, one not infrequently finds (percentage=6) that a typhoid serum that fails to give a positive Grünbaum-Widal reaction in a dilution of 1/30 may bring about agglutination in a dilution of 1/500 of the serum.

I merely offer this as a remotely possible explanation of certain of these negative phases, for phosphates with replaceable H groups are found in serum and as in presence of these a fair quantity of alkali is required to give the OH ion concentration necessary to produce inhibition, such an explanation is open to severe criticism, but the phosphates are present in small quantity only and constitute about 0.1 % of plasma by weight and as the alkalinity of the sera might be increased by contact with the soft glass of the capillary tubes in which the blood is collected, this explanation of the negative phase is quite possibly correct in a certain percentage of cases.

EXPERIMENTS ON THE INFLUENCE OF THE HYDROGEN ION ON AGGLUTINATION.

As the presence of hydroxidion has so marked an effect upon the phenomenon of clumping, we should expect, if the hypothesis that the inhibition is dependent upon alteration of the electrical state was correct, that hydrion would also induce an inhibition of agglutination.

That deviation from the isoelectric point leads also to inhibition if such deviation be on the acid side as well as if it be on the alkaline side is shown by the following experiments.

Technique. The procedure was much the same as that used in the previous series (Table IV), except that the concentrations of reagents employed were slightly modified. The organisms were sensitised with 1/100 serum, then washed twice, triturated, and agglutinated in presence of N/20 of the following electrolytes, varying proportions of N/50 HCl being added to each tube as shown in the following table.

TABLE VIII. 2 hours at 37°.

Electrolyte N/20	1 cc.	0.9 cc.	0.8 cc.	0.7 cc.	0.6 cc.	0.5 cc.	0.4 cc.	0.3 cc.	0.2 cc.	0.1 cc.	N/50 HCl
NaCl	-	-	-	-	-	-	-	-	-	-	-
Na ₂ SO ₄ ..	-	-	-	-	-	-	-	-	+	+	
Na ₃ Cit. ..	+	+	+	+	+	+	+	+	+	+	
BaCl ₂	-	-	-	-	-	-	-	-	-	-	-
Ce ₂ Cl ₅ N/2000	-	-	-	-	-	-	-	-	-	-	-

These results, which are the converse of those obtained in the corresponding experiment in which alkali was used to inhibit agglutination (Table IV), show the following striking phenomenon. The result obtained with Na₂SO₄ differs from that obtained with NaCl in that a greater concentration of the H ion is required to inhibit agglutination when the former salt is used as the precipitating electrolyte than when the latter is so employed. As both these salts are neutral, there is obviously some other factor than mere concentration of hydrion that must be taken into consideration. This point will be considered in dealing with the relation of the valency of the anion to the flocculation of sensitised bacteria suspended in acid fluids. Attention, however, may be drawn here to the absence of inhibition when citrate is employed which is probably due to two factors: (1) neutralisation of the acid, and (2) the valency of the anion.

That the effect of the acid is not dependent upon any permanent action upon the protoplasm of the organism was demonstrated as follows:

Technique. A saline suspension of the organism was exposed to the action of N/50 HCl for 2 hours at 37°, then centrifuged and washed twice and exposed to the action of serum in saline in the following dilutions.

TABLE IX. 2 hours at 37°.

	Dilutions of Serum						
	1/100	1/500	1/1000	1/2000	1/4000	1/8000	1/16000
Untreated organisms	+	+	+	+	+	-	-
Treated as described	+	+	+	+	+	-	-

That the acid has no permanent effect on the serum is apparent from the following experiment.

Technique. Serum in a dilution of 1/25 was exposed to the action of N/25 HCl in saline. This was then diluted (with saline) to produce the following concentrations of serum and acid with the addition of the bacillary emulsion to each tube.

TABLE X. 2 hours at 37°.

	Dilutions of Reagents						
	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400
Serum alone	+	+	+	+	+	+	-
Serum plus acid	-	-	-	+	+	+	-

Note. If the above mixtures are left in the incubator longer than 2 hours there is decided sedimentation in all the tubes, the precipitum however differs from true agglutination in that the flocculi are very small while the supernatant fluid remains markedly turbid.

From the experiments in the two preceding sections, the following conclusions may be drawn :

1. Agglutination is markedly affected by the reaction of the suspending fluid, and deviation—but with certain limits—from the “isoelectric point” results in inhibition of agglutination.
2. The inhibition is conditioned by the presence of hydrion and hydroxid-ion and does not result from permanent change produced in the protoplasm of the organism or the character of the serum by the action of such ions.
3. Agglutination probably depends upon the neutralisation of charge carried by particles in suspension.

EXPERIMENTS ON THE INFLUENCE OF THE VALENCY OF KATION AND ANION.

As previous experiments failed to show any variation in the activity—i.e. “titre”—of serum in presence of a variety of electrolytes whose solutions were approximately neutral and equimolecular with 0.9 % NaCl, the following experiment was carried out with a view to investigating the relative activity of a number of electrolytes acting upon sensitised organisms.

Technique. A 24 hours agar culture was washed off by saline and agglutinated at 22° for 18 hours in presence of 1/1000 agglutinating serum

(titre—1/8000). The supernatant fluid was pipetted off and the material was centrifuged and washed twice, then triturated and exposed to the action of the following salts.

TABLE XI. 2 hours at 37°.

		Dilution of Electrolytes					
		0.5 N	0.1 N	0.01 N	0.001 N	0.0001 N	0.00001 N
NaCl	..	+	+	+	-	-	-
Na ₂ SO ₄	..	+	+	+	-	-	-
Na ₃ Cit.	..	+	+	+	-	-	-
BaCl ₂	..	+	+	+	+	-	-
LaCl ₃	..	0	0	0	+	+	-

0=not done.

From these results it would seem that the valency of the kation of the electrolyte is the most important factor in conditioning the agglutination of sensitised organisms in the above experimental conditions.

Lest however the result obtained might have been due to a specific action of the salts employed, the following experiment with the salts of the alkaline earths was carried out.

TABLE XII. 2 hours at 37°.

		Dilution of Electrolytes														Normal
		0.005	0.004	0.003	0.002	0.001	0.0009	0.0008	0.0007	0.0006	0.0005	0.0004	0.0003	0.0002	0.0001	
NaCl	..	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
KCl	..	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
CaCl ₂	..	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
SrCl ₂	..	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
BaCl ₂	..	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
MgCl ₂	..	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-

This experiment shows that the result obtained cannot be explained on the hypothesis that it was due to any specificity of action of the salts employed.

These experiments agree with the findings of Coplans [1913] and of Beckhold [1904] that the agglutinating activity of an electrolyte depends upon the valency of its kation. The phenomenon is comparable too with the observations of Chick and Martin [1912] on the aggregation of particles of denaturated egg albumin. These authors show that egg protein dispersed by the addition of alkali to the suspending fluid is more susceptible to the action of salts with divalent kations than to those with monovalent kations while trivalent kations are more active than are divalent.

This, however, is not true of organisms that are suspended in acid fluids

and if agglutination be really comparable to the aggregation of denaturated protein one would not expect it to be so.

Moreover, if the process of sensitisation be akin to denaturation the anion should, in the case of acid suspensions, be the precipitating ion. To determine the validity of this hypothesis, the following experiment was carried out.

Technique. Organisms were agglutinated in saline for 2 hours at 37° in presence of 1/100 serum. These were then centrifuged and washed once in distilled water and then suspended in N/500 HCl. This suspension was added to equal volumes of the electrolytes so that the concentration of HCl in each tube was equal to N/1000, and the concentrations of the salts were such that with the addition of the suspension the following concentrations were obtained.

TABLE XIII. 2 hours at 37°.

		Dilutions					
		N/20	N/100	N/200	N/400	N/800	N/1600
NaCl ..	..	-	-	-	-	-	-
Na ₂ SO ₄ ..	..	+	+	+	+	+	+
Na ₃ Cit. ..	..	-	-	-	+	+	+
BaCl ₂ ..	..	-	-	-	-	-	-
Ce ₂ Cl ₅ ..	..	-	?	+	+	+	+

It will be seen that the result obtained is only in partial agreement with what one would expect, and the action of two of the salts requires special note.

(a) In the case of the citrate the absence of agglutination in presence of the higher concentration of the salt is probably due to the trivalent citrate ion conferring a strong negative charge upon the bacteria and so causing their dispersion.

That polyvalent ions may have such an effect is clear from the work of Burton [1906] on the influence of electrolytes upon the direction of movement of colloid particles exposed to the action of salts in an electric field. He found that the addition of aluminium ion to hydrosols of gold and silver in certain concentrations leads to the precipitation of the metal from its sol; lower concentration of the salts of aluminium did not have this effect and the particles, which in the sols of these metals bear a negative charge, continued to move in the same direction as before but at a diminished rate. If a concentration of aluminium ion greater than that required to bring about precipitation were employed, the particles remained dispersed, but

now their direction of movement in the field was reversed, indicating a reversal of charge carried by the particles of the sol.

In the case of copper sols, which carry a positive charge, exactly the opposite was noted—polyvalent anions in this case leading to the reversal of direction.

In the experiment described the effect of the citrate is apparently that of a polyvalent anion on a suspension bearing a negative charge. A positively charged and therefore dispersed protein (protein in acid suspension carries a positive charge) is precipitated in presence of certain concentrations of a salt with a trivalent anion, but with greater concentration of the same salt, owing to an overplus of negative charge being conferred upon the particles, they again become dispersed.

(b) The other point of special interest is the occurrence of a positive result with the cerium salt in acid suspension. I cannot suggest an adequate explanation of this peculiar finding but it will be seen from further experiments that salts with polyvalent kations behave in a manner very different from salts with mono- and di-valent kations in respect of their precipitation of bacterial suspensions.

In acid suspension as in alkaline suspension, then, sensitised bacteria behave as do denaturated protein particles and the results obtained agree in principle at least with those obtained by the authors above quoted [Hardy 1899; Chick and Martin 1912].

This finding further correlates the two phenomena, viz. aggregation of denaturated protein and agglutination of sensitised bacteria, so adding weight to my thesis that the process of sensitisation is similar to that of "denaturation."

FURTHER INVESTIGATION OF THE INFLUENCE OF THE VALENCY OF THE KATION.

The comparative action of Na, Ba, and La ions was next examined in further detail to determine if possible the quantitative relation existing between these ions in respect of their power of producing agglutination.

Technique. The organisms were sensitised as in the previous experiments and the concentrations of the salts were such that with the addition of the suspension the following concentrations were obtained.

TABLE XIV. 2 hours at 37°.

Sensitised Organisms													
	·1	·01	·005	·004	·003	·002	·001	·0009	·0008	·0007	·0006	·0005	·0004
NaCl	+	+	+	+	?	-	-	-	-	-	-	-	-
BaCl ₂	+	+	+	+	+	+	+	+	+	+	+	+	+
LaCl ₃	0	0	+	+	+	+	+	+	+	+	+	+	+
Unsensitised Organisms													
NaCl	-	-	-	-	-	-	-	-	-	-	-	-	-
BaCl ₂	-	-	-	-	-	-	-	-	-	-	-	-	-
LaCl ₃	0	0	?	+	+	+	+	+	+	+	+	+	+
Sensitised Organisms													
	·0003	·0002	·0001	·00009	·00008	·00007	·00006	·00005	·00004	·00003	·00002	·00001	Normal
NaCl	-	-	-	-	-	-	-	-	-	-	-	-	
BaCl ₂	?	-	-	-	-	-	-	-	-	-	-	-	
LaCl ₃	+	+	+	+	+	+	+	+	+	+	-	-	
Unsensitised Organisms													
NaCl	-	-	-	-	-	-	-	-	-	-	-	-	
BaCl ₂	-	-	-	-	-	-	-	-	-	-	-	-	
LaCl ₃	+	+	+	+	+	+	+	+	+	+	-	-	

0=not done.

This result is interesting for it bears some resemblance to the formula

$$1 : X : X^2$$

suggested by Linder and Picton [1895] for expressing the relative precipitating activity of mono-, di- and tri-valent kations acting upon colloidal As_2S_3 . The particles of this colloid, like organisms suspended in saline without previous treatment, carry a negative charge. The relative values of the kations as precipitating ions in the experiments of these investigators using the above colloid were

$$1 : 35 : 1023,$$

while in the above experiment taking the value for NaCl (0·003) as unity, the values of barium and lanthanum being 0·0003 and 0·00002 respectively, we obtain approximately

$$1 : 10 : 100.$$

Although this result is of considerable interest, I hesitate to lay any stress upon it, for the action of the salts with polyvalent kations is so different from that of other salts that they are not really comparable.

INVESTIGATION OF THE ACTION OF POLYVALENT KATIONS.

Gengou in an article on molecular adhesion [1908] points out that bacteria which have been exposed to the action of aluminium salts and washed in distilled water are agglutinated on the addition of NaCl. He says, "We

have also found that bacteria that have been agglutinated by moderate doses of alum and then washed in distilled water are well agglutinated on the addition of NaCl (this fact may be compared with the agglutination of bacteria by specific sera and with the observations of Beckhold [1904] on the flocculation of bacteria by the salts of heavy metals). If we treat bacteria that have been mixed with alum in a citrated medium in the same way, the subsequent agglutination by NaCl fails to occur. It is evident then that the mode of action of the citrate in this instance seems comparable to its action on substances in aqueous suspensions (barium sulphate, calcium fluoride, or mastic); but, owing to the fact that chemical reactions may take place between alum and citrate, and thus prevent the manifestations produced by the alum, we think it better to reserve any interpretation of this fact."

As Beckhold [1904] and other observers also call attention to similar phenomena, it seemed of interest to examine the action of salts with polyvalent kations on sensitised and unsensitised organisms.

Technique. (a) For the preparation of sensitised organisms, the growth was washed off into saline and exposed to the action of 1/500 serum (titre—1/8000) at 22° for 18 hours. The suspension was then centrifuged and washed with distilled water twice, then suspended in water and exposed to the action of the following electrolytes.

(b) For preparation of unsensitised organisms, the 24 hours growth was washed off with distilled water, then allowed to stand and centrifuged to obtain a deposit of organisms. The deposit was washed twice and a suspension in distilled water made as before.

Exposing these to the action of the following salts for 2 hours at 37°, agglutination took place thus :

TABLE XV. 2 hours at 37°.

	Sensitised Bacilli										
	N/20	N/40	N/100	N/200	N/500	N/1000	N/2000	N/5000	N/10000	N/20000	N/40000
NaCl	..	+	+	+	+	-	-	-	-	-	0
BaCl ₂	..	+	+	+	+	+	+	-	-	-	0
LaCl ₃	..	0	0	0	+	+	+	+	?	-	0
Ce ₂ Cl ₅	..	0	0	0	+	+	+	+	+	-	0
AlCl ₃	..	-	-	-	-	+	+	+	+	-	-
	Unsensitised Bacilli										
	N/20	N/40	N/100	N/200	N/500	N/1000	N/2000	N/5000	N/10000	N/20000	N/40000
NaCl	..	-	-	-	-	-	-	-	-	-	-
BaCl ₂	..	-	-	-	-	-	-	-	-	-	-
LaCl ₃	..	0	0	0	p	+	+	+	-	-	-
Ce ₂ Cl ₅	..	0	0	0	sl	sl	p	+	+	+	sl
AlCl ₃	..	-	-	-	-	-	+	+	+	+	-
0 = not done				p = partial			sl = very slight				

The salts with polyvalent kations, then, behave in a manner very different from the salts of mono- and di-valent kations—for they are apparently as active in agglutinating unsensitised organisms as in agglutinating sensitised organisms.

I hesitate to offer an explanation of this phenomenon, but it probably depends partly at least on the alteration of the sol form into the gel form of the hydroxides, as pointed out by Coplans [1913], for it is possible that the suspension of bacteria when prepared in the above manner has a slightly alkaline reaction, and this causing the formation of a gel form of the hydroxide gives rise to clumping by entanglement of the organisms in the contracting gel.

This would explain, in part at least, the negative phase obtained with the higher concentrations of AlCl_3 . The solution of this salt is acid owing to its hydrolytic decomposition, and the physical state of the hydroxide formed by adding the suspension will vary with the dilution of the salt, and as the agglutination by aluminium salts in all concentrations is inhibited by the presence of acid in the suspending fluid, this factor must be considered as a possible cause of the negative phase in the above experiment.

But the negative phase occurring with the higher concentrations of a polyvalent kation may be explained otherwise. It is possible that the polyvalent kations may produce a reversal of charge carried by organisms in the same way as it caused a reversal of direction of movement of the particles of metal sols of gold and silver in the experiments of Burton.

In order further to investigate this point TaCl_5 was made use of. This salt is very sparingly soluble in water and does not undergo hydrolysis with any concentration of the salt, therefore the addition of organism suspension should lead to the production of a hydroxide in the same physical state in each case, unless the concentration of the salt itself has a marked influence on the physical state of the hydroxide formed.

Employing the saturated solution of the salt, the following result was obtained.

TABLE XVI. 2 hours at 37°.

Sensitised Organisms							
TaCl_5 Sat. ..	1 cc	0.5 cc.	0.2 cc.	0.1 cc.	0.05 cc.	0.02 cc.	0.01 cc.
	—	—	+	+	—	—	—
Unsensitised Organisms							
TaCl_5 Sat. ..	—	p	+	+	—	—	—

This result shows that the negative phase in the case of polyvalent kations is not *wholly* explicable on the grounds that it is due to acid inhibition and it seems improbable—though I make the suggestion with reservation—that it is due in the case of tantalum to the formation of a hydroxide in a special state determined by the conditions of the experiment. In this connection the experiments of Gengou are of importance. The results obtained by that author suggest that, notwithstanding the fact that with TaCl_5 a negative phase does occur, the precipitating action of the polyvalent kations is partly explicable on the hypothesis that it is due to the formation of a hydroxide in accordance with the views of Gengou [1908].

The dispersion with the higher concentrations of the polyvalent kations may however be due to a reversal of charge produced by these reagents apart from that due to hydrolytic decomposition of the salts used.

In order to obtain further information on this point I decided to carry out the following experiment.

Technique. A large culture of the organism was washed off with saline and the suspension was divided into two parts, “a” and “b,” each of 5 cc. volume. To “a” was added agglutinating serum 1/500, while “b” was left unsensitised. After 18 hours at 22° the liquids were centrifuged, the deposit washed in distilled water, again centrifuged and the supernatant fluid decanted and replaced by N/250 AlCl_3 . Both were then allowed to interact for 2 hours at 37° . The liquids were again centrifuged and the deposit washed as before, and suspensions of the organisms were made in distilled water.

These suspensions were mixed (1) with NaCl, and (2) with NaCl in presence of N/200,000 $\text{Na}_3\text{Cit.}$, so that the concentrations shown in the following table were obtained in the tubes, when all the reagents were mixed.

Agglutination was allowed to proceed for 2 hours at 37° . The following results were obtained.

TABLE XVII. 2 hours at 37° .

		Sensitised Organisms						
		N/20	N/100	N/200	N/400	N/800	N/1600	NaCl
NaCl		..	+	+	+	+	+	+
NaCl plus citrate		..	-	-	-	-	-	-
Control		..	-	-	-	-	-	-
		Unsensitised Organisms						
NaCl		..	+	+	+	+	+	+
NaCl plus citrate		..	-	-	-	-	-	-
Control		..	-	-	-	-	-	-

Whether the polyvalent kations have a double action on the organisms is not clear. It might be that they act both as denaturing agents and as precipitating agents, and if this were so the agglutination by the polyvalent kations would be comparable to specific agglutination by sera in presence of mono- and di-valent ions.

If they do not act in this dual capacity, it is highly probable that the sedimentation is dependent on the formation of hydroxides.

I had hoped to obtain some light on the matter by performing the above test, but inasmuch as the sensitised and unsensitised organisms react in the same way when they have been exposed to the action of aluminium and subsequently agglutinated in presence of NaCl and of NaCl in citrate, no information can be gained upon the point.

Had the sensitised organisms agglutinated in presence of citrate after they had been exposed to aluminium, one could have stated that the action of the aluminium alone differed greatly from the action of serum in presence of NaCl or other electrolyte with mono- or di-valent kation.

The chief difficulty arises from the fact that the organism suspension adsorbs both serum and aluminium and the dispersion of the latter by the citrate masks or is more potent than the agglutination of the sensitised bacilli by the salt.

Apart then from the meagre indication obtained from the experiments in which TaCl_5 was made use of, evidence is lacking that the phenomenon of sedimentation by the polyvalent kations is not due to the formation of hydroxides of the metals in a peculiar physical state, and therefore agglutination of unsensitised organisms with these reagents is probably not comparable to agglutination in presence of specific serum.

Beyond calling attention to this phenomenon and submitting the above suggestions, I wish to advance no further hypothesis concerning the action of these bodies in producing agglutination of sensitised and unsensitised organisms, as it is highly probable that even a slight alteration in the conditions of the experiment would lead to very different results, for we are dealing with a number of reagents whose affinities for one another, whose physical state, and whose "concentration" under any circumstances are all mutually interdependent.

The only conclusion then that can be definitely deduced from this section of the investigation is that the salts with polyvalent kations behave in respect of both sensitised and unsensitised organisms in a manner very different from the salts with mono- and di-valent kations. The mechanism of their

action is obscure and one experiences difficulty in correlating the result obtained with aluminium and other trivalent ions with that obtained with the pentavalent tantalum.

EXPERIMENTS TO INVESTIGATE THE RELATION BETWEEN THE CONCENTRATIONS OF SERUM AND OF ELECTROLYTE.

Having determined that: (1) the precipitating value of an electrolyte acting on sensitised organisms is dependent on the valency of the kation of the electrolyte, and

(2) The titre of an agglutinating serum is no higher when a divalent kation is present in the precipitating fluid (BaCl_2 equimolecular with 0.9 % NaCl) than when a monovalent kation is employed (0.9 % NaCl) it seemed of interest to examine the relation of serum to electrolyte. It might well be that a low concentration of electrolyte would produce agglutination in presence of a given quantity of serum but that the reaction would not take place with the same concentration of the electrolyte in presence of less than that given quantity of the same serum.

To investigate this point, the following experiment was carried out.

Technique. An emulsion of organisms in distilled water was added to serum that had been diluted in NaCl and BaCl_2 to obtain the following concentration of the salts and dilutions of the serum when all the reagents were added to each tube.

TABLE XVIII. 2 hours at 37°.

NaCl					
N/20	N/40	N/100	N/200	N/400	Serum
+	+	+	+	+	1/100
+	+	+	+	+	1/500
+	+	+	+	?	1/1000
+	+	+	?	-	1/2000
+	+	?	-	-	1/4000
-	-	-	-	-	1/8000
BaCl_2					
N/400	N/500	N/666	N/1000	N/2000	
+	+	+	+	+	1/100
+	+	+	+	?	1/500
+	+	+	-	-	1/1000
+	+	-	-	-	1/2000
+	-	-	-	-	1/4000
-	-	-	-	-	1/8000

This shows that a relation of the nature indicated above does exist within certain limits; but the relation is probably not so simple as would appear from the above results, for BaCl_2 N/400 agglutinates in presence

of 1/4000 of the serum while in presence of BaCl_2 in concentration equal to N/6.5 (experiments of series 1) it only produces flocculation in 1/8000 of the same serum.

It is probable that as one approaches the dilution of serum which might be termed the "threshold value," the electrolyte has to be added in greatly increasing concentration in order to obtain aggregation of the bacteria.

SUGGESTED APPLICATION OF THE ABOVE TO THE DIFFERENTIATION OF BACTERIAL SPECIES.

It is possible that use might be made of the relation of serum to electrolyte for the purpose of differentiating closely allied bacterial species. Thus while the agglutinating "titre" of an anti-aertrycke serum might be the same for both *B. aertrycke* and for *B. paratyphosus b*, it seemed possible that if emulsions of these organisms were sensitised each in presence of 1/50 anti-aertrycke serum that the washed deposit in the case of the aertrycke bacilli sensitised with the anti-aertrycke serum might be flocculated by a lower concentration of the electrolyte employed than would the paratyphosus b. bacilli sensitised with similar concentration of the same serum. Or employing the serum as a constant throughout a series one might find that the anti-aertrycke serum in presence of aertrycke bacilli agglutinated with a lower concentration of electrolyte than did anti-aertrycke serum in presence of paratyphosus and *vice versa*.

The latter technique was adopted in the experiments. Employing anti-aertrycke serum in a dilution of 1/500 in presence of varying dilutions of NaCl to which were added emulsions of (1) typical *B. aertrycke*, (2) *Bacillus "D"*—a paratyphosus having peculiar reactions—and (3) typical *B. paratyphosus b*, the following result was obtained.

TABLE XIX. 1 hour at 37°.

Serum	NaCl	N/20	N/40	N/80	N/100	N/200	N/400
Anti-aertrycke	<i>B. aertrycke</i>	+	+	+	+	-	-
	<i>Bacillus "D"</i>	?	-	-	-	-	-
	<i>B. paratyphosus b</i>	?	-	-	-	-	-
	(titre of serum = 1/2000)						
Anti-paratyphosus b. ..	<i>B. aertrycke</i>	+	+	+	-	-	-
	<i>Bacillus "D"</i>	+	+	+	+	+	+
	<i>B. paratyphosus b</i>	+	+	+	+	+	+
	(titre of serum = 1/8000)						
Anti-"D"	<i>B. aertrycke</i>	+	+	+	-	-	-
	<i>Bacillus "D"</i>	+	+	+	+	+	+
	<i>B. paratyphosus b</i>	+	+	+	-	-	-
	(titre of serum = 1/4000)						

These results show that the specific antibody of the serum brings about agglutination in presence of a lower concentration of electrolyte than do the non-specific antibodies. Experiments with BaCl_2 corroborate the above results and I have applied the same technique in the examination of a number of strains of *B. aertrycke* and *B. paratyphosus b*, obtaining fairly constant results. The technique of the test, however, requires further elaboration before it could be satisfactorily applied to the differentiation of bacterial species.

INFLUENCE OF ACID AND ALKALI ON THE PROCESS OF "SENSITISATION."

The inhibiting action of alkalis and acids has been shown to be due to an interference with actual flocculation but it is possible that hydron and hydroxidion also affect the process of sensitisation.

To investigate the action of acid in this connection a mixture of organisms suspended in saline, with HCl present in concentration equal to N/100, was sensitised with specific agglutinating serum 1/100. The mixture was allowed to stand at room temperature for 48 hours, it was then centrifuged, the supernatant fluid drawn off and the deposit washed with distilled water. This procedure was repeated twice and the material obtained was then suspended in distilled water.

This emulsion was added to a series of tubes containing the following reagents of such concentration that with the addition of the bacillary emulsion the dilutions shown in the following table were obtained.

TABLE XX. 2 hours at 37°.

		N/20	N/100	N/1000	N/10000	N/100000
NaCl	..	-	-	-	-	-
Na_2SO_4	..	-	-	-	-	-
$\text{Na}_3\text{Cit.}$	..	-	-	-	-	-
BaCl_2	..	-	-	-	-	-

The above result appears to show that the presence of hydron interferes with the union of antibody with antigen in addition to inhibiting the actual process of clumping.

The converse of the above experiment was carried out thus :

Technique. A saline suspension of organisms was rendered alkaline by the addition of N/10 NaOH till the concentration of NaOH was equal to N/100. To this was added agglutinating serum sufficient to make the serum

equal to 1/50 of the fluid. The mixture was allowed to stand for 48 hours, and was then centrifuged, decanted and distilled water added. It was afterwards shaken and the washing process repeated twice. The suspension was then exposed to the action of NaCl of the following concentrations.

TABLE XXI. 2 hours at 37°.

	N/20	N/50	N/100	N/200	N/400
NaCl ..	-	-	-	-	-

From these results it would appear that the reaction of the fluid has a most important influence on the process of sensitisation and that if it be too alkaline or too acid, the process is inhibited just as flocculation is prevented by the presence of hydrion and hydroxidion in certain concentrations.

I do not wish to lay too great stress on this series of experiments since, while the above appears to be true in the experimental conditions of the test, it is quite possible that a slight alteration of the conditions might have a profound effect upon the result obtained, and as I have so far not had the opportunity of repeating the experiments of this series, I hesitate to draw any definite conclusion from them.

DISCUSSION OF RESULTS.

Reviewing the experiments that have been described, we see that, in the main, unsensitised organisms exhibit characters that are similar to those of fresh egg-white protein; sensitised organisms on the other hand recall in their properties certain features characteristic of "denaturated" egg-white. Unsensitised organisms and fresh egg protein behave as do "non-rigid" colloids, while the reactions of sensitised organisms and "denaturated" egg-white in presence of electrolytes recall those of "rigid" colloids under the influence of these reagents.

I advance the hypothesis then that the action of antisera is probably akin to that of denaturation and this raises the question—"Why is it that in the case of certain bacterial species sensitised (by specific serum) the organisms are flocculated by electrolytes, while in the case of other species they are not so flocculated?"

I cannot completely answer this question but if sensitisation be really akin to denaturation, we should almost expect that certain sensitised organisms would not be flocculated, for while denaturated egg-white is easily

flocculated by the addition of small quantities of electrolytes, serum protein on the contrary is very easily dispersed by the same reagents. The dispersion of egg-white is obtained only with difficulty by the use of salts and that only with polyvalent ions. I propose to investigate further this relation which I suggest exists between denaturation and sensitisation by performing a series of experiments similar to the above but employing anti-protein serum prepared by inoculating an animal with egg-white.

The fact that only certain serum-organism complexes show this peculiarity of flocculation is very strikingly shown in the case of the Bordet-Gengou bacillus of whooping cough [Bordet, 1909], in the case of which Bordet has, I think, shown conclusively that only certain antibody-antigen "complexes" are flocculable by electrolytes.

Looking at the question of bacteriolysis from the same standpoint, a number of important questions arise.

Thus, if the sensitisation of organisms to the agglutinating activity of electrolytes be a "denaturing" process, is the sensitisation of bacteria by specific serum to the dissolving activity of complement also a similar phenomenon? In fact does a process similar to denaturation precede solution in the process of bacteriolysis or cytolysis?

Arising out of the previous question a further problem presents itself—Is "complement" not a peculiar electro-physical state of some constituent or constituents of serum rather than a chemical "entity"?

I am at present engaged on the investigation of this problem, but so far I have not sufficient evidence for or against the hypothesis to permit me to draw any conclusions from the work done, beyond stating that salts with divalent ions inhibit the dissolving action of complement upon red corpuscles while they certainly have no inhibitory effect upon the process of sensitisation of these by haemolytic serum.

In conclusion I should like to call attention to the important question of specificity of action of sera in connection with what one might call the colloidal theory of immunity. Specificity is well explained certainly by the side-chain hypothesis of Ehrlich, but it seems to me that it is capable of explanation otherwise.

I hesitate to advance any theory on this point, but we see from the above experiments that in one respect certainly—that of flocculation—immunity reactions (in vitro) are very delicate indeed and in view of the fact that an electrolyte becomes, in general, a more potent disturbing factor with increase in complexity of the molecule, may it not be that with bodies of very large

molecule, like bacterial protoplasm and serum, the adjustment of the physical conditions of the interacting substances is exceedingly delicate and that upon the delicacy of this adjustment depend the phenomena of immunity ?

CONCLUSIONS.

The following conclusions seem permissible from the experiments described.

(1) That in presence of salts with mono- and di-valent kations unsensitised organisms behave in a manner similar to "non-rigid" colloids, but that when sensitised they behave in respect of these reagents in a manner similar to "rigid colloids."

(2) That the factors determining specific agglutination are markedly altered by the presence of hydron and hydroxidion in the suspending fluid.

(3) That the inhibitory action of these ions may be twofold, for they certainly inhibit flocculation and there is some evidence to show that they also interfere with the union of antigen with antibody.

(4) That—*caeteris paribus*—the character of the electrolyte has but little influence on the "titre" of the serum, provided always that it is sufficiently soluble and does not undergo hydrolysis.

(5) That when emulsions of bacteria that have been washed off directly in saline are used, the precipitating value of the electrolyte varies with the valency of the kation.

(6) That in acid suspensions, on the contrary, it varies with the valency of the anion.

(7) That the action of salts with polyvalent kations is peculiar in that these salts are as active in precipitating unsensitised organisms as they are in flocculating sensitised organisms.

No definite explanation of this is offered but it is suggested that the phenomenon of flocculation in these conditions depends upon a peculiar physical state of the metal hydroxides.

(8) That as unsensitised organisms behave in respect of the above points towards electrolytes as does fresh egg protein, but when sensitised have characters that recall those of denaturated egg-white, the process of sensitisation is akin to that of denaturation.

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