STUDIES IN AGGLUTINATION.

III. On the Mechanism of the Agglutination of Bacteria by Specific Agglutinating Serum.

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INTRODUCTION.

The first hypotheses concerning the laws governing immunological reactions were characterized by considerable complexity. The effort to describe these phenomena in chemical-physical terms has led to much clarification. In the reaction of bacterial agglutination, in particular, this type of approach has been most productive, and the earlier hypothetical explanations of the reaction have been gradually replaced by conceptions which conform with general chemical-physical laws.

Very early in the study of the phenomenon of specific bacterial agglutination, Bordet (1) made the observation that when bacteria are treated with homologous agglutinin, no flocculation is possible until electrolytes have been added. He thus conceived of specific agglutination as occurring in two phases, *first*, sensitization of bacteria by agglutinating serum (union of antibody and antigen) and *second*, flocculation of the agglutinin-bacteria complex by the electrolytes of the suspending fluid. He considered this flocculation analogous to the precipitation of colloidal suspensions by salts. That this two-phase conception is essentially correct has been abundantly confirmed.

First Phase.—Little is known of the reason for the selectivity of the interaction between agglutinin and homologous organism, *i.e.*, specific sensitization. The explanation will probably be forthcoming only when the exact chemical nature of the two reacting substances is determined.

The general character of agglutinin is known. It has been shown to be closely associated with the serum globulin, as it is precipitated out of the serum in the globulin fraction, and remains with the globulin (either euglobulin, pseudoglobulin, or both (2)) after purification of the protein. Also, the isoelectric point of the

agglutinin has been shown to be identical with that of globulin, pH 5.2–5.4 (3, 4). This has been disputed recently (5), but our own experiments support this conclusion. Agglutinin is not part of the serum albumin (2) and is not removed by lipoid extraction of the serum (6). That agglutinins are globulins is still unsettled; but that the agglutinin-globulin complex is the essential reacting substance seems clear.

Very important advances have been made recently by Avery, Heidelberger, and their coworkers (7) and Zinsser, Mueller, *et al.* (8), in the direction of ascertaining the chemical constitution of the portions of the bacterial cell which take part in specific interactions with antibody. The first group of workers have isolated from the three fixed types of pneumococcus and from strains of *B. friedländeri*, certain chemically distinct substances which they have shown to be polysaccharides, which react specifically with homologous antisera. These authors believe that these type-specific substances are to be found in the ectoplasmic layers of the cell and that, in view of the fact that the immune reactions are presumably cell surface phenomena, the presence in the periphery of the organisms of these substances determines the specificity and the readiness of response of the reaction. These workers have also isolated less clearly defined substances (nucleoproteins) which they have shown to be specific species-reacting substances. The second named investigators have shown that certain non-protein residue substances (carbohydrate gums) may be extracted from many types of bacteria and from yeasts, and that they bear a definite relation to the specific character of the organisms in that they react specifically with homologous immune sera.

To explain the mechanism of the union between antibody and organism, two main hypotheses have been suggested. The first, that the combination of the two is due to their possessing opposite electrical charges, seems to have been disposed of finally by Northrop and De Kruif (9) who have shown that union may occur when both elements are similarly charged.

The second explanation assumes that the antibody forms a film at the surface of the organisms and is held by ordinary forces of valence. A counterpart of this is found in the observation of Loeb (10) that collodion particles treated with proteins acquire a firmly fixed film of protein at their surfaces; a corollary, of much importance, in his work, is the observation that such film formation causes the particles to assume certain characteristics, notably the cataphoretic behaviour, of the proteins used.

In favour of the second hypothesis may be cited the following. Coulter (11) has shown that the optimum pH for agglutination of red blood cells is 4.75, but that when they are sensitized the optimum shifts to pH 5.3, *i.e.*, the isoelectric point of serum globulin. That is, in respect of their acid agglutination point, the red cells now behave like particles of euglobulin. Similar findings have been reported by Northrop and De Kruif (12) in experiments with bacteria and normal and immune sera, egg albumin and globin, and by Eggerth and Bellows (13) with bacteria and various proteins. That this assumption is correct seems to be established by certain of our experimental results that appear below.

Second Phase.—Much more is known of this aspect of the problem. Bechold (14), Neisser and Friedemann (15), and Arkwright (16), in support of Bordet, have emphasized the similarity between bacterial and colloidal suspensions in demonstrating the fact that bacteria possess an electrical charge, as evidenced by migration in an electrical field.[1] The two first named authors have carried the analogy further by demonstrating the similarity between the salt precipitation of kaolin and mastic, and of sensitized bacteria. They also have stated that sensitized bacteria lose their charge and have suggested that neutralization of charge is responsible for agglutination, recalling the observation of Hardy that suspensions of denatured protein (egg white) coagulate at the point at which they are devoid of charge; that this last assumption is partially incorrect appears later.

Tulloch (17) observed that, in respect of their behaviour toward electrolytes, unsensitized bacteria resemble fresh egg white and sensitized organisms resemble denatured egg white, *i.e.*, the former are precipitated by strong concentrations of salt only, while the latter flocculate readily in low salt, *e.g.*, physiological salt solution. He concludes that the process of sensitization is akin to denaturation of bacteria.

Buchanan (20), in an exhaustive review of the subject of agglutination, suggests that the like charges on bacteria act as forces of repulsion to keep the suspensions dispersed and that the force of surface tension acts as the attracting agency to cause agglutination. He suggests that agglutination occurs whenever factors are introduced which make the repelling force relatively less than the pull of surface tension.

The most valuable contribution to our knowledge of the chemical-physical laws governing bacterial agglutination is found in the recent work of Northrop and De Kruif (9, 12, 21). Their findings are so important, and so satisfactorily correlate the observations of previous investigators, that those conclusions bearing specifically upon the present consideration may well be reviewed at considerable length.

These authors accept the hypothesis that there are two factors making for repulsion, or for adhesion (and subsequent clumping) of bacterial particles; the first being the electrical charge on the organisms, and the second, a force, still insufficiently defined, to which they have assigned the name "cohesive force." They assume that, whenever the latter force is relatively higher than the former, agglutination results. However, they maintain that no satisfactory testing of these hypotheses is possible without measurement of both forces; accordingly they have devised a technique for measuring cohesive force. The method, as they indicate, is not one of absolute precision, but the values obtained are regularly reproducible; and in our hands (23) the method has given perfectly satisfactory results. Bacterial charge was measured from the rate of migration in a cataphoretic cell.

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[1] For theoretical discussions of the origin of this charge see the work of Loeb (10, 18), Northrop and De Kruif (21), and Winslow, Falk, and Caulfield (19).

Their conclusions may be summarized briefly as follows: (1) Agglutination is to be considered in terms of two antagonistic forces: a *repelling force*, due to like electrical charge, which tends to keep the bacteria apart, and *"cohesive force,"* which makes for adhesion. In any bacterial suspension, all factors that make the repelling force relatively greater than the cohesive force make for stability; and conversely, all factors that reduce the repelling force or otherwise make the cohesive force relatively greater, lead to flocculation. (2) In the case of unsensitized bacteria, electrolytes in lower concentrations, < 0.01 N, affect primarily the potential, and in higher concentrations, > 0.01–0.1 N, affect primarily the cohesive force. (3) As long as cohesive force is unaffected, agglutination occurs whenever the charge is reduced by electrolytes to a point below a critical level of about 15 millivolts;[2] that is, the unaffected cohesive force now becomes relatively greater than the force of repulsion. (4) Salt in high concentration depresses the cohesive force of unsensitized bacteria so that no agglutination occurs even though there may be no measurable charge; *i.e.*, the cohesive force is now so small that it is always less than the repelling force. (5) When bacteria are treated with immune sera, their cohesive force is in some manner protected from this depressing effect of strong salt (*e.g.*, physiological salt solution, etc.) and agglutination is determined solely by the charge; that is, whenever the potential of the sensitized bacteria is reduced by electrolyte to a point below 15 millivolts, the suspension agglutinates. This explains the observation of Bordet, confirmed by Northrop and De Kruif in the course of the work being cited, that electrolytes are essential for specific agglutination. That is to say, the salt, routinely used in the ordinary reactions, reduces the charge on the bacteria so that this charge comes to lie in the 15 millivolt agglutination zone, and, the cohesive force of the sensitized bacteria, being insusceptible to the depressing effect of the electrolyte, is now relatively greater than the repelling force and flocculation occurs.[3] (6) Their results refute the idea that combination of antibody and organism is caused by difference of sign of the charges carried by the two substances; but are in agreement with the assumption that the agglutinin forms a film on the surface of the organism.

In the course of studies concerned with the importance of environmental factors in agglutination (23), we have been much interested in the nature of the changes in electrical charge that occur when bacteria are treated with agglutinative sera. As a result of observations upon these changes, much light has been shed upon the general nature

[2] Loeb (22) has shown that a similar critical potential agglutinating zone exists for many varieties of suspended particles, *cf.*, native and denatured proteins, collodion, graphite, etc.

[3] That this may not be the only part played by electrolytes is indicated, however, in experiments still under investigation, with organisms having a low primary charge, less than 15 millivolts.

of the reaction, and we now feel that we have sufficient data upon which to found a satisfactory hypothesis for the mechanism of the agglutination of bacteria by specific serum.

Methods.

Pseudoglobulin was obtained from human ascitic fluid as follows: the globulin was thrown down with 50 per cent $(NH_4)_2 SO_4$ and the precipitate put into membranes and dialyzed against running tap water 48 hours. To this, $(NH_4)_2 SO_4$ to make 25 per cent, was added to remove the euglobulin—to the filtrate enough salt was added to make 50 per cent and the pseudoglobulin precipitate was redialyzed against tap and distilled water at pH 5.4. The precipitate in this was discarded. The resulting solution of pseudoglobulin was 4.5 per cent.

All other methods used, unless otherwise indicated, are similar to those described in the preceding paper (24).

EXPERIMENTAL RESULTS.

The first step in specific bacterial agglutination is selective interaction between agglutinin and organism. As will be recalled, the evidence thus far obtained favours the assumption that this union consists of a coating of the organism by agglutinin. Proof of the validity of this assumption is essential to the hypothesis for the mechanism of the reaction to be outlined below. The following experiments seem to establish the correctness of this conception of sensitization.

In a preceding paper (24), it has been shown that in the cases of Type I pneumococcus and *B. paratyphosus* A, their agglutinative sera possess a specific charge-reducing effect which is quantitatively related to the titer of the serum. It has been shown that this effect disappears following absorption of agglutinin, and that it is not present in highly protective sera that have no agglutinative properties.[4]

Fig. 1 illustrates this effect in the case of the Type I pneumococcus serum. It will be seen from this figure that both normal and immune sera depress the charge on the bacteria as their concentration is increased, but it will be observed that the normal serum effect is only slight while the reducing effect of the specific serum is striking.

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[4] We have since observed also that the protective antibody of Huntoon (25), which is practically non-agglutinative, does not show this specific effect.
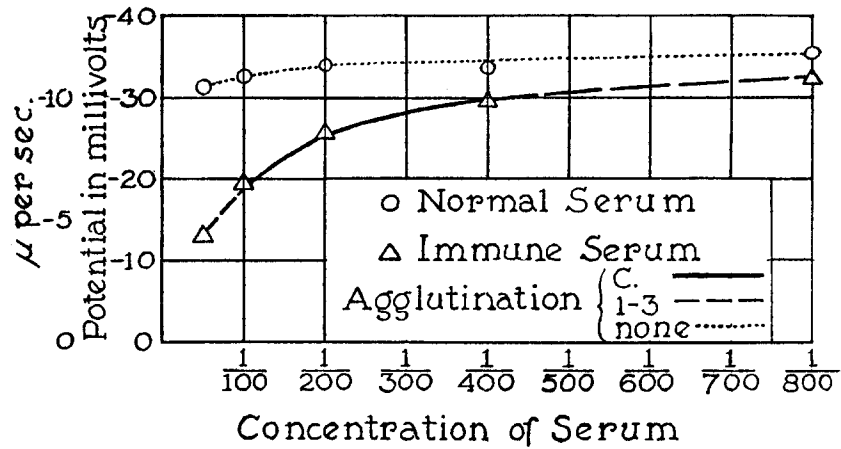
FIG. 1. Effect of normal serum and Type I pneumococcus serum on potential and agglutination of Type I pneumococcus. Experiment in м/200 glycocoll-phosphate-acetate buffer, pH 7.0. In this and subsequent figures complete agglutination is shown by a solid line, incomplete by a broken line, and absent agglutination by a dotted line. This figure is kindly reprinted from the previous paper (24).
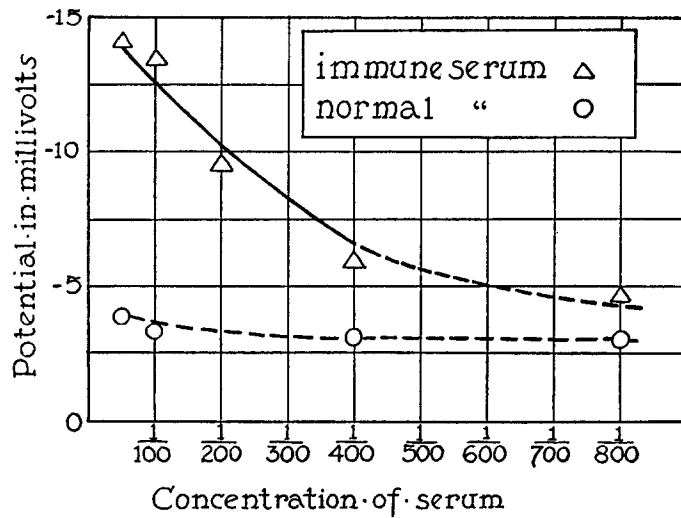

FIG. 2. Effect of normal serum and specific agglutinative serum upon *B. dysenteriæ*, Shiga strain. There was no agglutination by normal serum. Experiment in м/200 glycocoll-phosphate-acetate buffer, pH 7.0.

We have since extended observations of this phenomenon to several other bacteria and their agglutinating sera. The organisms studied now include, Type I pneumococcus, meningococcus, staphylococcus, *Streptococcus scarlatinæ*, a diplococcus variant of a hemolytic streptococcus, *B. coli*, *B. typhosus*, *B. paratyphosus* A, *B. tuberculosis*, and three strains of the dysentery bacillus, Flexner, Shiga, and Mt. Desert.

Confirmation of the fact that agglutinative sera possess this charge-reducing effect was obtained in all cases studied except the three dysentery strains. In the case of these organisms, an unexpected result was secured when it was found that their specific sera in high concentration not only do not reduce the charge, but raise it. Fig. 2 illustrates this effect in the case of the Shiga strain and its agglutinative serum.

When the specific effects of all the agglutinative sera upon the cataphoretic charges of their homologous organisms are plotted upon the same chart (Fig. 3), the explanation of this paradoxical behaviour becomes evident. Reference to this figure brings out the fact that all the organisms other than the dysentery strains have, under the conditions of the experiment, high or relatively high charges (23 to 40 millivolts) when untreated with serum; while the three dysentery strains possess relatively low charges (3 to 5 millivolts). It will be seen that in the case of the first group cited there is specific charge reduction, quantitatively related to the agglutinative titer, and that in the second group there is a similar charge elevation. In all cases, however, no matter what the initial charge (range is 5–40 millivolts) high concentrations of serum (over 1:50), bring the charges on all bacteria to a *common potential level* (8–14 millivolts).

In other words, when different bacteria are treated with homologous agglutinative sera in high concentration, they behave cataphoretically alike, irrespective of their previous differences in charge. This suggests that they are alike because they have a similar surface coating. To put it in another way, when bacteria are sensitized by agglutinative sera, a film of agglutinin (globulin) is formed on their surfaces and such sensitized bacteria, in the sense of Loeb's protein-coated particles discussed above, all behave cataphoretically like particles of globulin.

It is worth calling attention, at this point, to the work of Northrop and Freund (26) on the agglutination of red blood cells. They have

found that the potential agglutination zone of unsensitized cells is low (below 6 millivolts) and that, when sensitized the critical point is raised to about 12 millivolts. This is approximately the critical point for globulins, as will be seen below. Accordingly, the difference between the unsensitized and the sensitized cells may well be accounted for on the assumption that the latter are coated with specific antibody (globulin).
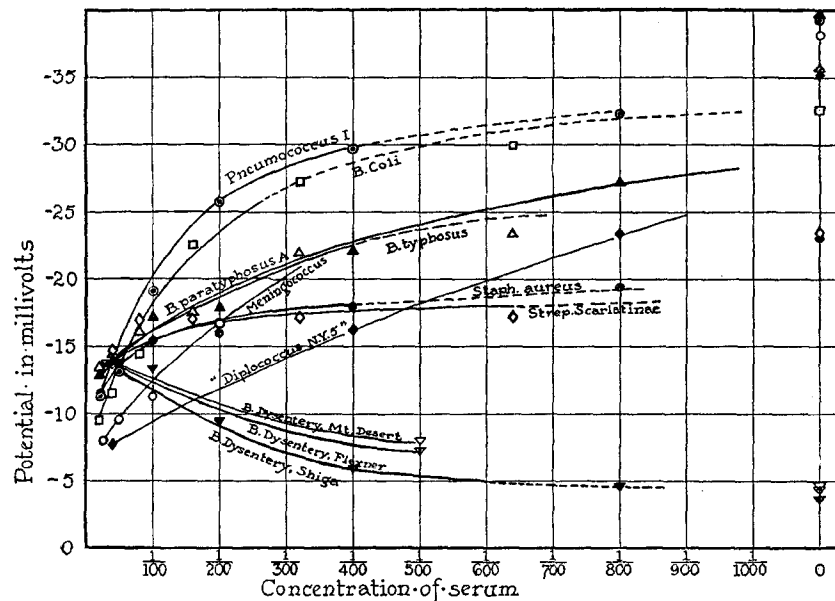

Fig. 3. Effect of eleven specific agglutinating sera upon the potential and agglutination of their homologous organisms. These experiments were done over a period of about 2 years and three different cataphoretic cells were utilized in the work. All experiments in m/200 glycocoll-phosphate-acetate buffer mixture, pH 7.0.

That the conception of sensitization as specific coating with globulin is correct is made still more clear by the evidence afforded in the following experiments.

The cataphoretic behaviour of sensitized Type I pneumococcus was compared with that of the globulin from the homologous serum as follows: The euglobulin of the serum was precipitated by 1:20 dilution with distilled water. The particles thus obtained have protective

antibody (Felton (27)), and agglutinin, as demonstrated by control experiments; they are readily visible microscopically and are thus susceptible of study in an electrophoretic cell. The bacteria were sensitized by treatment with 1:20 immune serum. Unsensitized organisms and organisms treated with 1:20 normal serum were used as controls. The four sets of particles were now added to varying concentrations ($M/25$–$M/800$) of glycocoll-phosphate-acetate buffer (21) of pH 7.0, and their charges determined. The results are shown in Fig. 4. The upper curve is that of the untreated organisms, and
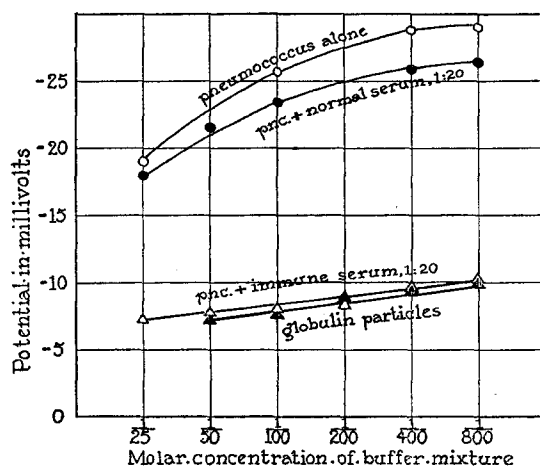

FIG. 4. Effect of glycocoll-phosphate-acetate buffer, pH 7.0, in varying molar concentration, upon potential of Pneumococcus Type I, (a) alone, (b) treated with 1 /20 normal and (c) with 1 /20 specific serum, and (d) of globulin particles obtained from the specific serum.

the next, that of those treated with normal serum, showing the usual slight charge reduction. The two lower curves are those with which we are chiefly concerned. It will be seen that the curve of the sensitized organisms is practically identical with that of the globulin particles. That is, the former are acting cataphoretically as if they were globulin, a fact which can be explained only on the assumption that they are coated with globulin.

The cataphoretic charges of sensitized organisms were next compared with those of particles of denatured pseudoglobulin in solutions

of varying hydrogen ion concentration. The bacteria were colon bacilli treated with 1:20 homologous agglutinating serum, centrifuged, and then resuspended in the solutions in which they were to be tested. The pseudoglobulin, non-specific, was denatured by bringing it, in dilute solution, to the boiling point; the resultant flocculant mass was centrifuged, divided into small homogeneous particles in a mortar, and added to the solutions of varying pH. Unsensitized colon bacilli were tested similarly for comparison. The results appear in Fig. 5.
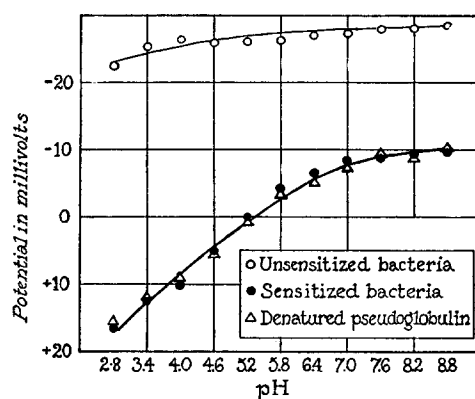

Fig. 5. Effect of varying pH upon potential of unsensitized and sensitized colon bacilli and of particles of denatured pseudoglobulin. Experiment with glycocoll-phosphate-acetate buffer, M/25. Agglutination of sensitized bacteria and pseudoglobulin was complete throughout and was absent in case of unsensitized colon bacilli.

It will be observed that the cataphoretic behaviour of the sensitized bacteria is quite unlike that of the unsensitized organisms, but it is practically identical with that of the denatured pseudoglobulin particles. In other words, the sensitized organisms behave cataphoretically as if they are particles of globulin. A finding again accounted for by the assumption that the organisms are coated with agglutinin.

It will be noted also, from this chart, that the isoelectric point of the sensitized bacteria is the same as that of the pseudoglobulin, which is the accepted one of approximately pH 5.3. This is in accord with the findings of Coulter, Northrop and De Kruif, and Eggerth and Bellows, already cited.

The evidence presented above seems to lead reasonably safely to the conclusion that the first step in specific bacterial agglutination is a selective film formation at the surface of the organism by the globulin of the antibody, and that the changes in charge on the bacteria are the result of this coating.   In high concentrations of homologous agglutinating serum, the coating by globulin is complete and the organisms behave exactly like particles of globulin.   When the serum is dilute, the degree of coating is less and the charges observed represent the potential difference between the surrounding medium and a combination of bacterial surface and agglutinin film.

What degree of coating is essential for agglutination of the bacteria we are not prepared to say, as the data for such estimation are difficult to obtain.   Northrop and De Kruif believed from their experiments, that if film formation could be assumed, it could be calculated that agglutination occurs when the surface is one-eighth covered.   From examination of our results with sera having a high agglutinative titer, it is our impression that the amount of coating required may well be less than this.

The further interpretation of the part played by this selective coating of the bacteria in specific agglutination is based largely upon certain observations and conclusions of Loeb (10, 18, 22) in his work with collodion particles and proteins.   It is necessary therefore, to consider briefly those portions of his work that bear upon our problem.

Loeb treated collodion particles with various proteins, egg albumin, gelatin, casein, edestin, and found that a durable film of these proteins is formed at the surface of the particles.   He showed that such coated particles now behave cataphoretically as if they are no longer collodion particles but are particles of the protein used.

He observed further, that in respect of their stability in suspension, the coated particles (except those treated with gelatin) no longer behave like soluble native proteins which are precipitated only by very strong salt, but act like denatured proteins, which are relatively unstable and are easily thrown out of suspension by electrolytes in low concentration.   That is to say, the chemical forces of attraction between the "aqueous" groups of the protein molecule and the water are destroyed in the film formation, as is the case when protein is denatured by boiling, and the stability of the coated particles depends only, as does that of denatured proteins, upon the electrical double layer surrounding each particle (i.e., the charge).   To recapitulate, collodion particles coated with protein no longer act like collodion particles, but behave as if they are particles of denatured protein, and flocculate

as soon as their charge is reduced by electrolyte to a critical point of about 13 millivolts.

It is important to note, that in the case of particles of denatured protein, the cohesive force (in the sense used for bacteria, by Northrop and De Kruif) is practically insusceptible to the reduction effect of high concentrations of salt. This is true also for protein-coated particles.

These findings of Loeb supply the reason for the observed fact that sensitized bacteria behave like particles of denatured protein. That is, when the organism is coated with agglutinin and assumes the character of a globulin particle, by virtue of this very film formation, it takes on the character of denatured globulin. Because of this similarity, Tulloch, as noted above, believed that sensitization is akin to denaturation of bacteria. However, the bacterial surface itself is not "denatured," but the film of globulin formed at its surface assumes the characteristics of denatured protein, and the coated organism now acts like a particle of denatured globulin. The same author's analogy between native protein and unsensitized organisms, also noted above, is probably incorrect, as the behaviour of the former is dependent upon true solubility and the failure of the latter to flocculate is due to reduced cohesive force, as shown by Northrop and De Kruif.

The observation of Northrop and De Kruif that the cohesive force of sensitized bacteria is protected against reduction by strong salt is also accounted for. The explanation lies in the fact that the surface film of globulin, having the character of denatured protein, is insusceptible to this depressing effect, and that while electrolytes may reduce the charge on the coated particles, they do not affect their cohesive force, and agglutination is determined solely by reduction of the potential to the critical 15 millivolt level.

Before proceeding to full application of these findings of Loeb to specific agglutination, it seemed necessary to prove that the behaviour of globulins with collodion particles is similar to that of the proteins used by Loeb. Accordingly, certain of his experiments were repeated with pseudoglobulin.

The procedure followed was essentially that described by Loeb (10, 18, 22). Collodion particles were coated with isoelectric pseudoglobulin. Particles of denatured pseudoglobulin were prepared by bringing a dilute solution to the boiling point, centrifuging the floc-

culant mass, and dividing it into suitable homogeneous particles in a mortar. The effect of three electrolytes, NaCl, $K_4Fe(CN)_6$, and $CeCl_3$, upon the charge and agglutination of untreated collodion particles, coated particles, and particles of denatured pseudoglobulin was determined at pH 3.0.[5] The results, shown in Fig. 6, demonstrate the fact that pseudoglobulin behaves in similar fashion to the proteins used by Loeb. Reference to the figure shows that collodion particles treated with pseudoglobulins no longer behave like the untreated
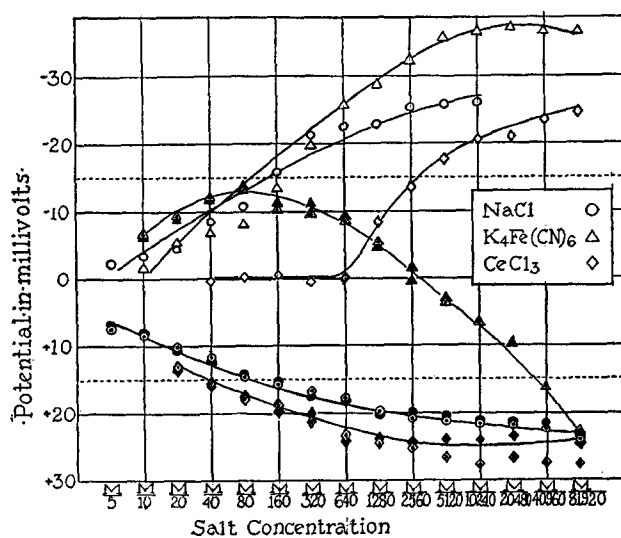

FIG. 6. Influence of NaCl, $K_4Fe(CN)_6$, and $CeCl_3$ upon potential and agglutination of collodion particles (blank symbol), collodion particles coated with pseudoglobulin (symbol with solid center), and particles of denatured pseudoglobulin (solid symbol). Experiment at pH 3.0. Critical potential agglutination zone was between +13 to +15 millivolts and −13 to −15 millivolts. In this zone all particles flocculated out of suspension.

particles, but assume the cataphoretic charges and the critical potential agglutination level (14 millivolts) of the denatured protein. That is to say, they now behave, in these respects, as if they are particles of denatured pseudoglobulin.

[5] The experiments were conducted at this pH because Loeb believed that coating occurred only on the acid side of the isoelectric point of the protein used. Dr. Northrop, in a personal communication, states that evidence has been obtained in his laboratory that coating occurs on the alkaline side as well.

SUMMARY.

From the foregoing evidence, specific bacterial agglutination may be conceived of as follows: When bacteria are mixed with their homologous agglutinative sera, specific union between organism and agglutinin occurs. This interaction consists of specific coating of the bacteria by globulin. By virtue of the fact, noted by Loeb and illustrated in Fig. 6, that protein film formation gives the coated particle the characteristics of denat ured protein, the bacteria now take on the character of particles of denatured globulin. Particles of denatured protein flocculate whenever their charge is reduced by electrolyte to a critical level lying somewhere between 12 and 14 millivolts. This is true even when the salt is strong, as, unlike bacterial particles, their cohesive force is not readily depressed by salt. The sensitized bacteria, now being essentially particles of denatured protein, likewise agglutinate as soon as their charge is reduced by electrolyte to this potential level.

CONCLUSIONS.

1. In the process of sensitization by agglutinating serum bacteria are coated selectively by the globulin of the antibody.

2. This film formation causes the bacteria to take on the characteristics of particles of denatured globulin.

3. Subsequent agglutination of the coated bacteria follows the laws governing the flocculation of particles of denatured protein by electrolytes.

BIBLIOGRAPHY.

1. Bordet, J., *Ann. Inst. Pasteur*, 1896, x, 193; 1899, xiii, 225.
2. Kapsenberg, G., *Z. Immunitätsforsch.*, 1925, xlii, 284.
3. Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1912, xlvii, 59.
4. von Szent-gyorgi, A., *Biochem. Z.*, 1921, cxiii, 36.
5. Ottenberg, R., and Stenbuch, F. A., *J. Gen. Physiol.*, 1925–26, ix, 345.
6. Hartley, D., *Brit. J. Exp. Path.*, 1925, vi, 180.
7. Avery, O. T., and Heidelberger, M., *J. Exp. Med.*, 1923, xxxviii, 73, 81; 1924, xl, 301; 1925, xlii, 377; Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1925, xlii, 355; Heidelberger, M., Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1925, xlii, 727.

8. Zinsser, H., *J. Exp. Med.*, 1921, xxxiv, 495; Zinsser, H., and Parker, J. T., *J. Exp. Med.*, 1923, xxxvii, 275; Mueller, J. H., and Tomcsik, J., *J. Exp. Med.*, 1924, xl, 343; Zinsser, H., and Mueller, J. H., *J. Exp. Med.*, 1925, xli, 159; Mueller, J. H., Wayman, M., and Zinsser, H., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 241; Mueller, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 209; Mueller, J. H., Smith, D. E., and Litarczek, S., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 373.

9. Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1922–23, v, 127.

10. Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 109, 395.

11. Coulter, C. B., *J. Gen. Physiol.*, 1920–21, iii, 307.

12. Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 655.

13. Eggerth, A. H., and Bellows, M., *J. Gen. Physiol.*, 1921–22, iv, 669.

14. Bechold, H., *Z. physik. Chem.*, 1904, xlviii, 385.

15. Neisser, M., and Friedemann, U., *Münch. med. Woch.*, 1904, li, 465, 827.

16. Arkwright, J. A., *J. Hyg.*, 1914, xiv, 261.

17. Tulloch, W. J., *Biochem. J.*, 1914, viii, 293.

18. Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 479; 1923–24, vi, 105.

19. Winslow, C.-E. A., Falk, I. S., and Caulfield, M. F., *J. Gen. Physiol.*, 1923–24, vi, 177.

20. Buchanan, R. E., *J. Bact.*, 1919, iv, 73.

21. Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 639; 1922–23, v, 127, 139, 605; *J. Exp. Med.*, 1923, xxxvii, 647.

22. Loeb, J., *J. Gen. Physiol.*, 1923–24, vi, 215.

23. Shibley, G. S., *J. Exp. Med.*, 1924, xxxix, 245.

24. Shibley, G. S., *J. Exp. Med.*, 1924, xl, 453.

25. Huntoon, F. M., *J. Immunol.*, 1921, vi, 117, 123, 235.

26. Northrop, J. H., and Freund, J., *J. Gen. Physiol.*, 1923–24, vi, 603.

27. Felton, L. D., *Boston Med. and Surg. J.*, 1924, cxc, 819.