# THE STABILITY OF BACTERIAL SUSPENSIONS.

## III. Agglutination in the Presence of Proteins, Normal Serum, and Immune Serum.

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It has frequently been noted that the addition of a small amount of certain substances, especially proteins, markedly affects the behavior of suspensions. It was found by Whitney and Blake[1] for instance that the sign of the charge of gold particles in the presence of gelatin could be reversed by acids, a result which did not occur without gelatin. The same effect has been noted by Loeb[2] in the case of collodion membranes treated with different proteins. The membrane always acquires the isoelectric point of the protein used. It was found by one of the writers that peptone markedly affects the acid agglutination of the bacillus of rabbit septicemia. It has been shown in the preceding paper[3] that the isoelectric point was also displaced. This result had been noted by Putter.[4] The present paper contains the results of experiments on the effect of proteins and sera on the properties of suspensions of bacteria.

Fig. 1 shows the effect of various concentrations of egg albumin on the agglutination and charge of the bacillus of rabbit septicemia (Type D strain). The method of plotting is the same as in the preceding paper. Increasing the amount of egg albumin gradually shifts the curve to the alkaline side so that the isoelectric point is moved to pH 5.0 which is approximately that of egg albumin. In other

[1] Whitney, W. R., and Blake, J. C., *J. Am. Chem. Soc.*, 1904, xxvi, 1339.

[2] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 659; 1921–22, iv, 213.

[3] Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 639.

[4] Putter, E., *Z. Immunitätsforsch., Orig.*, 1921, xxxii, 538. The same observation had been made independently by one of the writers De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 345.

words, the particles act more and more like particles of egg albumin. The form of the curve is very similar to the curve found by Loeb[5] for the potential between a solution of egg albumin in a collodion sac and the surrounding solution. As was found in the experiments described in the preceding paper, agglutination occurs whenever the potential is reduced below a value of about 15 millivolts. The result of the addition of egg albumin is, therefore, that the agglutination zone is moved to the alkaline side and that at a pH of 3 the egg albumin stabilizes the suspension instead of precipitating it.

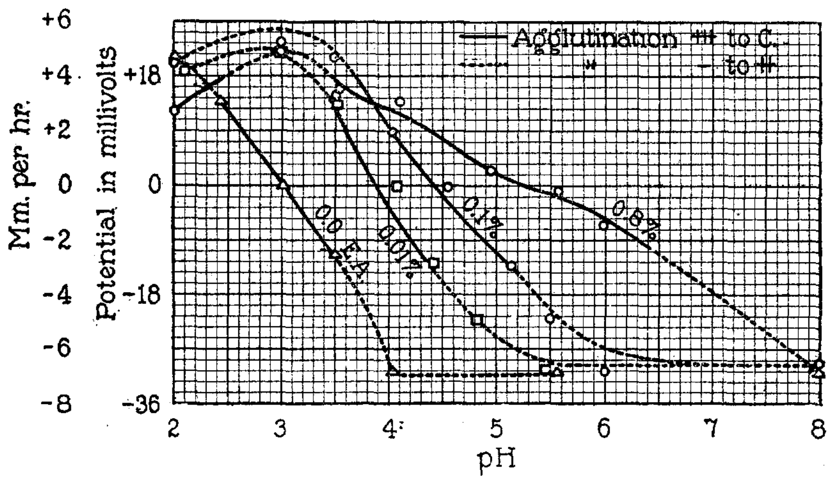


FIG. 1. Effect of the concentration of egg albumin at different pH on the potential and agglutination of Type D. pH adjusted with acetate buffers.

This is typical of the action of protective colloids and is due, as the figure shows, to the increase in the potential. The figure also shows that the amount of egg albumin required to agglutinate is a minimum near the isoelectric point of the suspension and increases as the pH is moved to the alkaline side. Similar experiments have been published by Eggerth and Bellows.[6]

Fig. 2 shows the effect of the addition of globin to a suspension of Type D; the isoelectric point is now shifted to pH 6.5 which is near

[5] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922.

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

the isoelectric point of globin. Agglutination again occurs whenever the potential is less than 15 millivolts.

The effect of normal and immune serum on the pH curves of *Bacillus typhosus* is shown in Fig. 3.[7] The result is very similar to egg albumin. It will be noted that there is no marked difference between the immune and the normal serum and also that the isoelectric point is shifted to a pH of 4.7 in both cases. This was an unexpected result, since the isoelectric point of blood globulin is given by Mi-

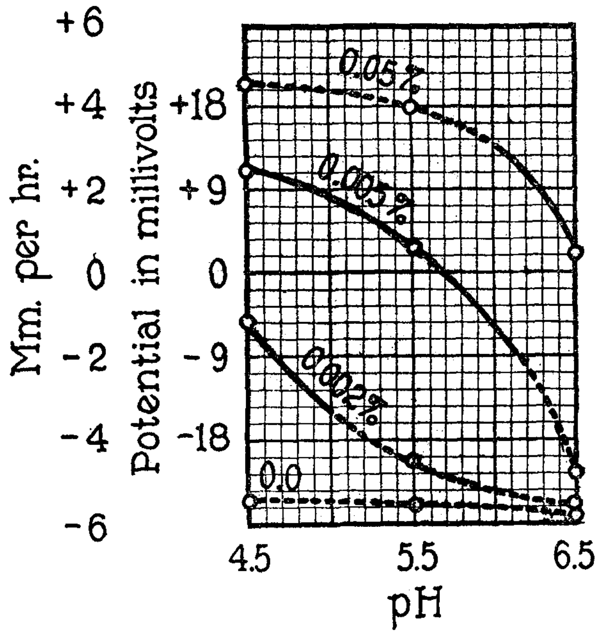


FIG. 2. Effect of the concentration of globin on the potential and agglutination of Type D at different pH. Acetate buffers.

chaelis[8] as 5.4 and the antibodies are known to be associated with the globulins. According to the present experiment, however, the substance in the serum which has the greatest effect on the charge of the

[7] Similar experiments have been made by Kōsaka and M. Seki, Communication to the Okayama Medical Society, *Okayama-Igakkwai Zasshi*, 1922, No. 386.

[8] Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914. It is doubtful if this can be considered the isoelectric point of pure immune body since traces of foreign proteins have such a marked effect.

organisms has an isoelectric point at about pH 4.7. The small difference in the concentration of normal and immune serum required to change the isoelectric point renders it improbable that this effect can be ascribed to the immune body.

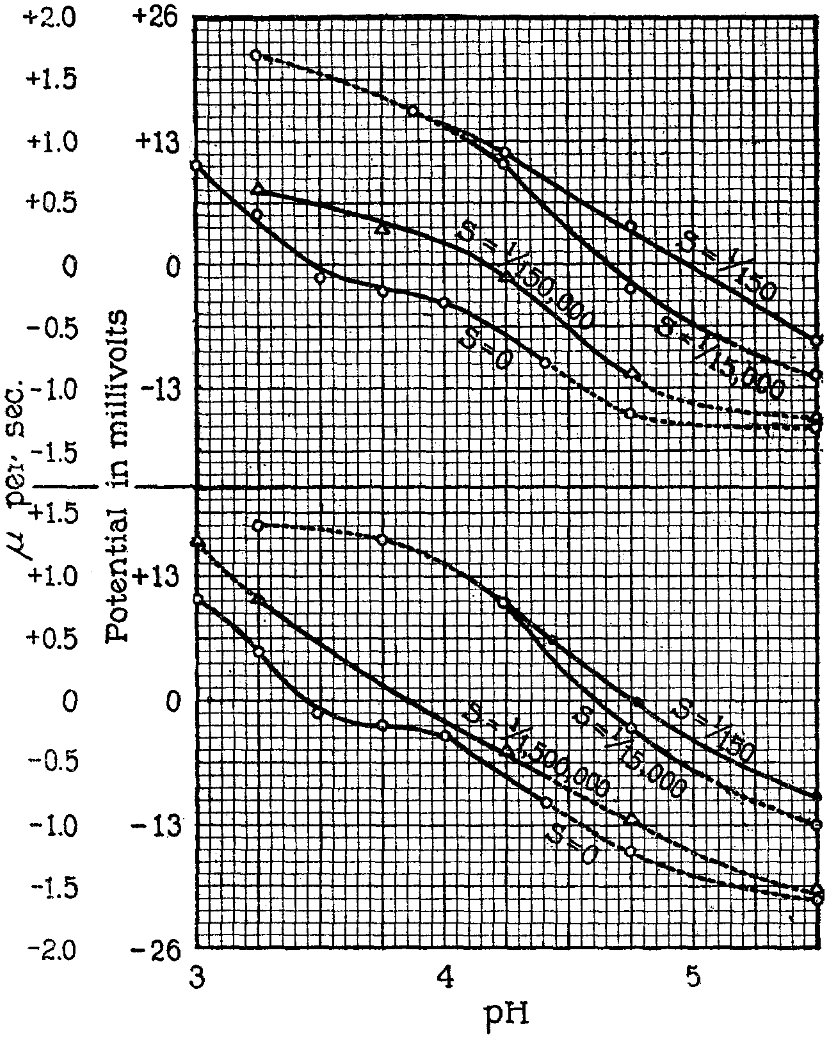


FIG. 3. Effect of different concentrations of normal (upper half) and immune serum (lower half) on the potential and agglutination of *B. typhosus* at different pH 0.01 N acetate buffer.

The stabilizing effect of the serum at pH 3 is likewise due to some constitutent of the serum other than the immune body as is shown by the following experiment. A suspension of *Bacillus typhosus* was treated with an excess of immune serum in 0.10 N salt and then washed once with distilled water. The suspension was then added to pH 3 buffer. The result was instantaneous and intense agglutination, whereas Fig. 3 shows that in the presence of excess serum no agglutination occurs at a pH of 3.

TABLE I.

*Agglutination of B. typhosus by Antityphoid Horse Serum at Various* $C_H$.
*G. P. A. Buffer.*

| pH | Concentration of serum. | | | | | | | | Control. No serum. |
|---|---|---|---|---|---|---|---|---|---|
| | $5 \times 10^{-4}$ | $2.5 \times 10^{-4}$ | $1.25 \times 10^{-4}$ | $6.2 \times 10^{-5}$ | $3.1 \times 10^{-5}$ | $1.55 \times 10^{-5}$ | $7.8 \times 10^{-6}$ | $3.9 \times 10^{-6}$ | |
| 8.5 | C. | C. | C. | C. | C. | ++ | – | – | – |
| 7.5 | C. | C. | C. | C. | C. | ++ | + | – | – |
| 6.0 | C. | C. | C. | C. | C. | ++ | + | – | – |
| 5.5 | C. | C. | C. | C. | C. | ++ | + | – | – |
| 5.2 | C. | C. | C. | C. | C. | C. | + | Tr. | – |
| 5.0 | C. | C. | C. | C. | C. | C. | ++ | + | – |
| 4.6 | C. | C. | C. | C. | C. | C. | C. | C. | Tr. + |
| 3.9 | C. | C. | C. | C. | C. | C. | C. | C. | C. |
| 3.3 | + | + | + | ++ | ++ | C. | C. | C. | C. |
| 2.7 | + | + | + | + | + | + | + | + | + |

*Normal Horse Serum.*

| | pH | $2 \times 10^{-3}$ | $1 \times 10^{-3}$ | $5 \times 10^{-4}$ | $2.5 \times 10^{-4}$ | $1.25 \times 10^{-4}$ | $0.6 \times 10^{-4}$ |
|---|---|---|---|---|---|---|---|
| Normal horse serum 1.0 cc. + typhoid suspensions 1.0 cc. | 5.0 | Tr. | Tr. | – | – | – | – |
| | 4.7 | + | Tr. | Tr. | – | – | – |
| | 4.4 | + | + | Tr. | + | ++ | ++ |
| | 3.9 | – | Tr. | + | C. | C. | C. |
| | 3.3 | – | – | + | + | ++ | ++ |

Tables I to IV show the effect of the pH on the amount of normal serum and immune serum to cause agglutination of various organisms.

TABLE II.

*Agglutination of Types I and II Pneumococci by Pneumococcus Type I Antiserum at Various $C_H$. G. P. A. Buffer.*

| | pH | Dilution of serum. | | | | | | | | | Control. No serum. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1,280 | 1:2,560 | |
| | 8.5 | C. | C. | C. | ++ | Tr. | – | – | – | – | – |
| Pneumococcus | 7.1 | C. | C. | C. | ++ | + | – | – | – | – | – |
| Type I serum | 6.0 | C. | C. | C. | ++ | + | – | – | – | – | – |
| dilutions 1.0 cc. | 5.5 | C. | C. | C. | ++ | + | – | – | – | – | – |
| + pneumococcus | 5.2 | C. | C. | C. | C. | + | + | + | Tr. | Tr. | – |
| Type I suspen- | 5.0 | C. | C. | C. | C. | ++ | + | + | + | Tr. | – |
| sions 1.0 cc. | 4.6 | ++ | ++ | C. | C. | C. | C. | ++ | + | Tr. | Tr. |
| | 3.9 | + | + | + | + | + | ++ | C. | C. | C. | C. |
| | 8.5 | + | + | – | – | – | – | – | – | – | – |
| Pneumococcus | 7.1 | + | + | Tr. | – | – | – | – | – | – | – |
| Type I serum | 6.0 | ++ | ++ | + | Tr. | – | – | – | – | – | – |
| dilutions 1.0 cc. | 5.5 | ++ | ++ | + | Tr. | – | – | – | – | – | – |
| + pneumococcus | 5.2 | ++ | ++ | + | Tr. | Tr. | – | – | – | – | – |
| Type II suspen- | 5.0 | ++ | ++ | + | + | Tr. | Tr. | – | – | – | – |
| sions 1.0 cc. | 4.6 | C. | C. | C. | ++ | + | + | Tr. | Tr. | Tr. | – |
| | 3.9 | + | + | C. | C. | C. | C. | C. | ++ | + | + |

TABLE III.

*Agglutination of Type G Rabbit Septicemia Bacillus by Rabbit >G at Various $C_H$.*

| pH | Dilution of serum. | | | | | | | | | | | Control. No serum. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1:10* | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1,280 | 1:2,560 | 1:5,120 | 1:10,240 | |
| 7.9 | ++ | ++ | + | + | – | – | – | – | – | – | – | – |
| 7.5 | ++ | C.† | ++ | + | Tr. | – | – | – | – | – | – | – |
| 7.0 | ++ | C. | C. | ++ | ++ | + | Tr. | – | – | – | – | – |
| 6.5 | C. | C. | C. | C. | ++ | + | + | Tr. | Tr. | Tr. | Tr. | Tr. |
| 6.0 | C. | C. | C. | C. | C. | ++ | + | + | Tr. | Tr. | Tr. | Tr. |
| 5.5 | C. | C. | C. | C. | C. | C. | ++ | ++ | + | + | + | + |
| 5.0 | C. | C. | C. | C. | C. | C. | C. | ++ | ++ | ++ | C. | C. |
| 4.5 | + | + | + | C. | C. | C. | C. | C. | C. | C. | C. | C. |
| 2.2 | – | – | – | – | – | – | – | – | – | – | – | – |

* Represent *serum dilutions*. Divide by 2 to obtain final dilution.

† C = Complete agglutination.

++ = Strong agglutination with sediment but turbid supernatant.

\+ = Slight agglutination.

Tr. = Trace.

TABLE IV.

| pH | Dilution of normal serum. | | | | | | | | | | Control. No serum. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1,280 | 1:2,560 | 1:5,120 | |
| 7.0 | + | + | – | – | – | – | – | – | – | – | – |
| 6.0 | + | + | + | + | + | + | Tr. | Tr. | Tr. | Tr. | Tr. |
| 5.5 | + | + | + | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| 5.0 | + | ++ | ++ | ++ | ++ | C. | C. | C. | C. | C. | C. |
| 4.5 | ++ | ++ | ++ | ++ | ++ | C. | C. | C. | C. | C. | C. |
| 4.0 | + | + | ++ | ++ | ++ | C. | C. | C. | C. | C. | C. |
| 3.0 | + | + | + | + | + | ++ | ++ | ++ | ++ | ++ | C. |
| 2.0 | – | – | – | – | – | – | Tr. | Tr. | Tr. | Tr. | Tr. |

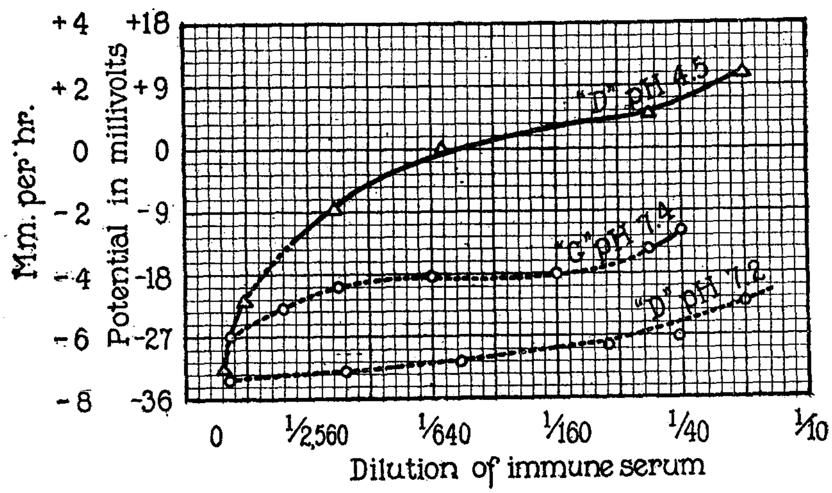


FIG. 4. Effect of immune serum on the potential and agglutination of Type D and Type G at different pH. G. P. A. Buffer.

The results are the same as with egg albumin. In every case the acid agglutination zone is shifted to the alkaline side, and the amount of serum required to agglutinate is a minimum near the isoelectric point of the organism. This result had been noted by Krumwiede and Pratt,[9] and by Michaelis and Davidsohn.[10] It is noticeable also that

[9] Krumwiede, C. K., Jr., and Pratt, J., *Z. Immunitätsforsch., Orig.*, 1913, xvi, 517.

[10] Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1912, xlvii, 59.

the difference between the normal and the immune serum becomes less and less marked as the pH approaches that of the acid agglutination zone of the organism.

Fig. 4 shows the effect of immune serum on the charge and agglutination of Types D and G. As in all the experiments, the agglutination becomes complete as soon as the charge is reduced below 15 millivolts. The figure shows that Type D is difficult to agglutinate because it has a fairly high charge at a pH of 7.2 and the effect of the immune serum is insufficient to reduce this to the critical value. Type G, however, has a lower charge and is much more readily agglutinated. Type D at a pH of 4.5 is easily agglutinated since at this pH the serum has a much greater effect on the charge.

### *The Effect of Salts.*

Bordet[11] showed that salt greatly increased agglutination with immune serum. Porges,[12] however, found that with very powerful immune serum agglutination occurred even though the serum was dialyzed and no salt was present.

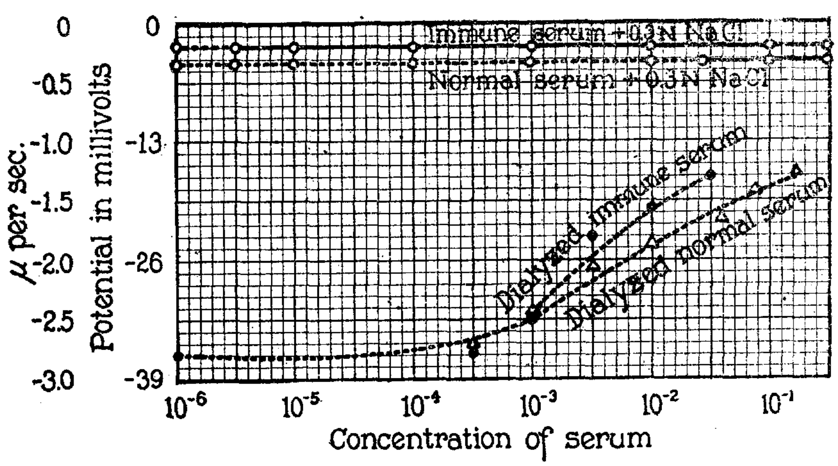


FIG. 5. Effect of dialyzed normal and immune serum on the potential and agglutination of *B. typhosus*. Upper curves show the effect in the presence of 0.3 N NaCl. The potential in these two curves is identical but they have been separated slightly in the figure in order to show the difference in the agglutination.

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[11] Bordet, J., Traité de l'immunité dans les maladies infectieuses, Paris, 1920.

[12] Porges, O., *Centr. Bakt.*, *1 te Abt.*, *Orig.*, 1905, xl, 133.

The effect of dialyzed normal and of powerful antityphoid horse serum on the potential and agglutination of *Bacillus typhosus* is given in Fig. 5. The two upper curves are the results in 0.3 N NaCl. There was no complete agglutination in the absence of salt and no marked difference between the normal and immune serum, although both affect the potential. (The serum was prepared by dialysis against distilled water and then dissolved by the addition of a small amount of NaOH. Conductivity measurements showed that the total concentration of salt was less than 0.001 N; *i.e.*, too small to cause the noted effect on the potential.) In the presence of salt, on the other hand, there is no effect on the potential but agglutination occurs in very high dilution with the immune serum and to a much less extent with the normal serum. This experiment shows that the effect of the serum on a suspension of bacteria in *concentrated salt* solution is not primarily on the charge but on the cohesive force. The serum raises the cohesive force and hence the critical charge to a value greater than the potential carried by the organism and they therefore agglutinate. The effect of the serum on the cohesion is shown in Fig. 6. The upper part of the figure shows that the addition of serum raises the cohesion to the value in distilled water; *i.e.*, it prevents the salt from decreasing the attractive force and thereby lowering the critical potential. The lower part of the figure shows the converse experiment; *i.e.*, the effect of salt on a film of washed, and of sensitized organisms. The salt decreases the cohesion of the washed organisms very markedly but has no effect on the cohesion of the film sensitized with serum.

The effect of varying both the salt and the serum concentration on the agglutination is shown in Table V. As the serum concentration is increased, the salt concentration in which complete agglutination occurs widens on both sides from 0.10 N. The lower limiting concentration of salt remains at about 0.01 N, however, and does not continue decreasing as the serum increases. In other words, the effect is not additive, but there is a critical concentration of serum beyond which there is little or no effect on the concentration of salt needed to agglutinate. This "critical" salt concentration corresponds to the point at which the charge on the organisms is about 10 millivolts; *i.e.*, just under the critical potential. This is the result expected if the agglutinin forms a film on the surface of the organism. As soon as the layer is complete the addition of excess serum will have no effect. If this assumption is correct, it follows from Table V that agglutina-

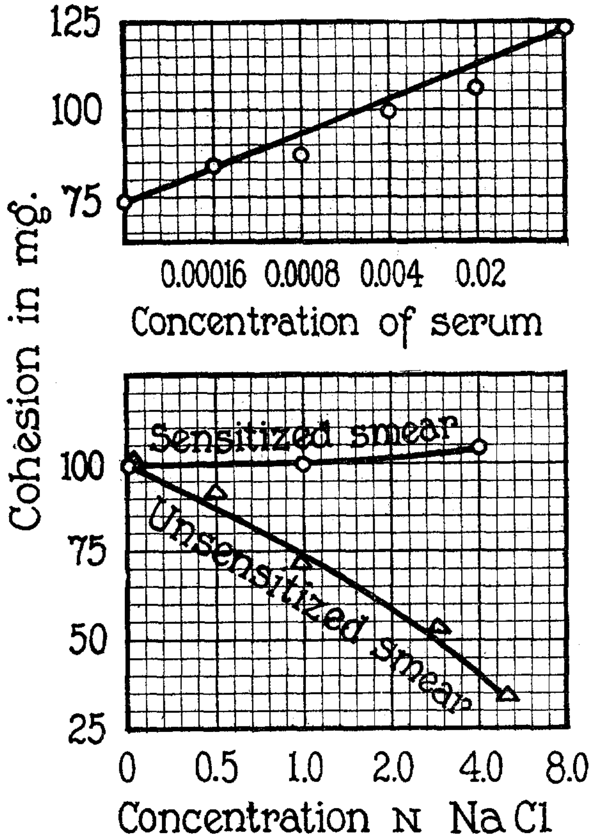


FIG. 6. Upper half, effect of the concentration of immune serum on the cohesive force between two films of *B. typhosus* in 0.10 N NaCl. Lower half, effect of concentration of NaCl on the cohesive force between, a, smears treated with immune serum and, b, untreated smears.

tion occurs when the surface is about one-eighth covered. The inhibiting effect of strong salt solution is, as usual, due to the decrease in the cohesive force, when the organisms are not completely covered with immune body.

It is evident from the foregoing that the agglutination may be considered as caused by the salt, as Bordet stated. The serum, however, does not *sensitize* the bacteria but *protects* it from the salt so that the latter does not reduce the cohesive force. If we study the effect of salts and acids on the agglutination and charge of organisms sensitized with immune serum, we should expect then to obtain curves similar

TABLE V.

*Influence of NaCl Concentration on Agglutination with Dialyzed Normal and Immune Serum.*

| Concentration of immune serum | Agglutination after 24 hrs. at 20°C. | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Concentration of NaCl. | | | | | | | | |
| | 0 | 0.001 | 0.003 | 0.01 | 0.03 | 0.1 | 0.3 | 1.0 | 1.4 |
| 1:150 | + | + | +++ | C. | C. | C. | C. | C. | C. |
| 1:300 | − | − | +++ | C. | C. | C. | C. | C. | C. |
| 1:600 | − | − | +++ | C. | C. | C. | C. | C. | C. |
| 1:1,200 | − | − | ++ | C. | C. | C. | C. | C. | C. |
| 1:2,400 | − | − | + | C. | C. | C. | C. | C. | ++ |
| 1:4,800 | − | − | − | ++ | C. | C. | C. | C. | + |
| 1:9,600 | − | − | − | + | ++ | C. | C. | ++ | − |
| 1:19,200 | − | − | − | − | + | C. | ++ | + | − |
| 0 | − | − | − | − | − | + | + | − | − |
| Concentration of normal serum. | | | | | | | | | |
| 1:12 | + | ++ | +++ | C. | C. | C. | C. | C. | C. |
| 1:24 | − | − | + | C. | C. | C. | C. | +++ | +++ |
| 1:48 | − | − | − | +++ | +++ | +++ | ++ | + | + |
| 1:96 | − | − | − | ++ | +++ | ++ | + | + | − |
| 1:192 | − | − | − | − | + | ++ | + | − | − |
| μ per sec. | −2.5 | −1.3 | −1.0 | −0.7 | −0.4 | −0.15 | 0 | 0 | 0 |
| Millivolt potential. | −32 | −17 | −13 | −9.0 | −5.6 | −0.19 | 0 | 0 | 0 |

to those given in the preceding paper with the exception that the stable zone in high concentrations of salt would not appear and the agglutination should be found to depend entirely on the potential. A summary of a number of such experiments is given in Fig. 7. The serum concentration was 1:500 in all cases; *i.e.*, in excess. The figure shows that with the exception of strong acid solution, complete agglutination occurred whenever the potential was reduced below 15

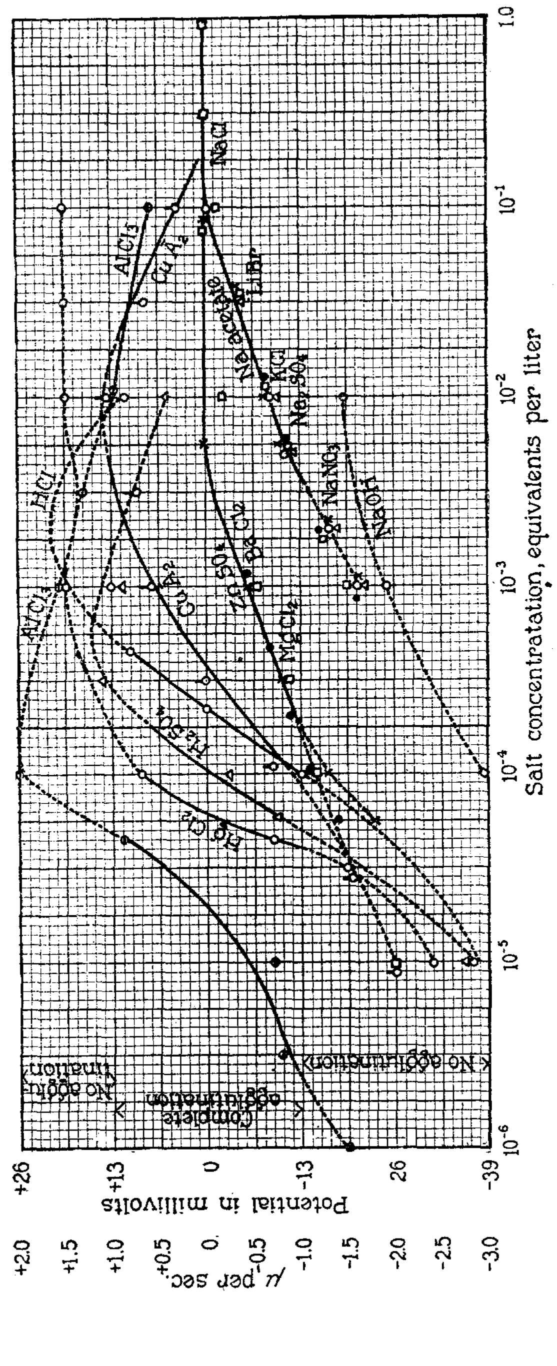


FIG. 7. Effect of the salt concentration on the agglutination and potential of *B. typhosus*, sensitized with immune serum.

millivolts, and there is no stable zone in concentrated salt. The effect of the strong acid is due partly to destruction of the antibody and partly to the fact that the combination of the antibody with the bacteria is less complete in acid solutions.[13]

The results also show that the effect of all monovalent cations (except hydrogen) was identical both as regards potential and agglutination. The valency and nature of the anion have no effect. This is the usual result when the particles are negative. The bivalent cations agglutinate in much lower concentration. The trivalent curves are not comparable owing to changes in the pH.

## SUMMARY.

1. The addition of proteins or serum to suspensions of bacteria, (*Bacillus typhosus* or rabbit septicemia) at different pH widens the acid agglutination zone and shifts the isoelectric point to that of the added substance.

2. The amount of serum required to agglutinate is much less near the acid agglutination point of the organisms.

3. The addition of immune serum prevents the salt from decreasing the cohesive force between the organisms, and agglutination therefore is determined solely by the potential, provided excess immune body is present. Whenever the potential is decreased below 15 millivolts the suspension agglutinates.

[13] This point is taken up in the following paper.