THE STABILITY OF BACTERIAL SUSPENSIONS.

II. THE AGGLUTINATION OF THE BACILLUS OF RABBIT SEPTICEMIA AND OF BACILLUS TYPHOSUS BY ELECTROLYTES.

BY JOHN H. NORTHROP AND PAUL H. DE KRUIF.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 22, 1922.)

It is a very old observation that the stability of suspensions is markedly affected by the addition of electrolytes. Under certain conditions the particles remain separate, while under other conditions they adhere to each other. In the latter case the particles settle rapidly and the suspension is said to be coagulated or agglutinated. Since under certain conditions the particles remain distinct and in others collect into large masses, it is evident that there is a force which tends to hold them together and another force which tends to keep them apart. If the attractive force is greater than the repulsive force, the particles agglutinate. It was early found that nearly all substances in suspension are electrically charged with reference to the surrounding liquid, and it was suggested by Jevons that the repulsion due to this charge was the repelling force. This conception was substantiated by Hardy, who found that suspensions of denatured proteins coagulated at the point at which they carried no electric charge. Hardy called this the isoelectric point. Hardy's experiments have been greatly extended by Michaelis and his coworkers. It is probable, however, that the precipitation of proteins and the agglutina-
tion of suspensions are governed by entirely distinct forces. In the case of oil emulsions Powis was able to show that agglutination occurred whenever the potential became less than a certain critical value, in this case about 30 millivolts. Powis' experiments leave little doubt that the potential at the oil-water surface is the determining factor in the agglutination of oil emulsions. Burton also found that metallic suspensions coagulate in the zone where the potential is small, although he did not find such a definite critical value.

In the case of bacteria, however, the results have been much less satisfactory. It was found by Bechhold, Arkwright, Teague and Buxton, and others that bacteria were always negatively charged whether or not they were agglutinated. These authors concluded, therefore, that the potential carried by the organisms could not account for the phenomena. Putter was able to show some qualitative agreement between the potential and agglutination of Bacillus typhosus.

Results of the Present Experiments.

It is evident that in order to test the hypothesis outlined above, it is necessary to measure both the force which tends to cause the particles to adhere as well as that which keeps them apart, since if both forces are affected by the conditions of the experiment but only one is measured, it will be impossible to interpret the results. The potential may be conveniently measured by the rate of migration in an electric field. The attractive forces, however, have usually been assumed to remain constant and no attempt has been made to measure them. It was found, in the course of the present experiments, that a comparative measure of the attractive forces between the organisms could be obtained by measuring the force required to tear apart two glass plates covered with a film of the bacteria and immersed in the solution which was under investigation. As a result of

7 Putter, E., Z. Immunitätsforsch., 1 te Abt., Orig., 1921, xxxii, 538.
these measurements in conjunction with the measurements of the potential difference, it has been found that whenever the potential difference between the surface of the bacteria and the solution is less than about 15 millivolts the bacteria agglutinate, provided the cohesive force is not affected. If the cohesive force is decreased, this critical potential is decreased, and if the cohesive force is made very small, no agglutination occurs even though the potential be reduced to zero. It was further found that all electrolytes tested in concentrations less than 0.01 to 0.1 N affect primarily the potential, while in concentrations greater than 0.1 N the effect is principally on the cohesive force. In the case of bacteria sensitized with immune serum, the cohesive force remains constant and the agglutination can be predicted solely from the measurement of the potential.

Experimental Methods.

Measurement of the Potential.—The potential was determined from the rate of migration as described in the preceding papers. The U-tube method was used for the experiments with the bacillus of rabbit septicemia and the microscopic method with B. typhosus.

Measurement of the Cohesive Force.—A piece of thick glass slide was covered with a thin film of very heavy suspension of washed organisms (B. typhosus), the film allowed to dry and then heated to 60° for a few minutes. This causes the bacteria to adhere firmly to the glass. A heavy (No. 3) cover-slip was similarly prepared. The cover-slip was suspended by means of a fine platinum wire from the lever of the du Noüy9 surface tension apparatus. The glass slide was immersed in a dish containing the solution to be studied and the cover-slip allowed to rest on it with its own weight for 1 minute. The force required to pull the cover-slip from the slide was then determined. It was found that if the measurement was made immediately after the two surfaces came in contact, the value obtained depended on the force with which the two had been pressed together. If the slip had been pressed down firmly a much greater force was required than if it had simply been allowed to rest on the slide. After a short time interval, however, this difference became less, and eventually the same reading was obtained in both cases. This is due presumably to the fact that the distance apart of the two surfaces is regulated by capillary forces and comes to the same point from either side. The same smear was used as long as the same value was obtained on replacing the preparation in distilled water. The value obtained becomes less after ten or

fifteen measurements due to the gradual removal of the film. Control experiments with clean glass surfaces showed no significant variation under the conditions of the experiment. The values obtained in this way were surprisingly reproducible. They have been expressed as milligrams required to separate two surfaces each 2 cm. square. The results are not exactly comparable to the measurements of the potential since the organisms have been subjected to dry heat. It will be noted, in fact, that the results do not conform exactly to those expected from the potential measurements. In the case of NaCl, for instance, the concentration required to affect the cohesive force noticeably, is slightly higher than would be expected from the potential curve.

It has usually been considered that this force is a surface tension effect, but there does not appear to be any conclusive evidence as to its nature. It is better, perhaps, to refer to it simply as "cohesive" without an exact definition of its nature.

**Measurement and Regulation of the Hydrogen Ion Concentration.**—The pH determinations were made electrometrically, using a saturated calomel cell and taking the pH value of 0.10 N HCl as 1.04 at 33° as the standard.

**Buffers Used.**—It was found that a very convenient buffer could be made by combining sodium phosphate, sodium acetate, and glycine. It may be used over a range of pH from 1 to 13 and has the further advantage that the nature of ions present is not varied. The only variation is a change in concentration of the Cl and Na ions. The composition and the titration curve of the buffer are given in Fig. 1. This is referred to as G. P. A. Buffer. The pH measurements were made at 33°. In some experiments Walpole’s acetate series was used.

**Cultures.**—The culture of the bacillus of rabbit septicemia used was that previously isolated and described by one of the writers.11 The typhoid culture was the Pfeiffer strain obtained through the kindness of Dr. Charles Krumwiede to whom we are also indebted for the strong antityphoid horse serum.

**Measurement of the Degree of Agglutination.**—No satisfactory method could be found for measuring the agglutination quantitatively. Several degrees of agglutination were, therefore, selected and the determinations made on this basis. They were recorded as follows:

- No agglutination.
- + Distinct particles visible with a lens, 8 diameters magnification.
- ++ Particles visible with the eye alone.
- +++ Suspension almost completely agglutinated and settled but cloudy appearance in the supernatant liquid.
- C. Supernatant liquid perfectly clear.

The stage marked C. is the easiest to detect with certainty and was used as the end-point.

The degree of agglutination increases with time at first but after 24 hours remains constant. All readings were therefore made after 24 hours at 20° to elimi-

---

Fig. 1. Titration curve of glycine acetate phosphate buffer.
nate the time factor. A typical experiment is shown in Table I. It is evident that there is some relation between the charge and the rate of agglutination. The suspensions having the lowest charge are the ones which agglutinate the most rapidly. The table shows, however, that the relation is not continuous. Those suspensions having a potential greater than about 15 millivolts do not agglutinate completely at any time. In other words, the potential does not merely effect the time required for agglutination, but if larger than a certain value, prevents it entirely. This is the result obtained by Powis. The fact that the point of agglutination is not sharp but covers a fairly wide range between no agglutination and complete agglutination, may be due to the individual variation in the particles. It would be better theoretically, therefore, to use the point of half coagulation as the end-point. This cannot be determined experimentally owing to the lack of a quantitative method for determining the degree of agglutination.

**Preparation of the Suspension.**—It has been noted by one of us\textsuperscript{12} that the presence of traces of peptones, etc., present in the culture medium, markedly affect the agglutination of the organisms. The suspensions were therefore thoroughly washed in distilled water. 24 hour broth cultures of the organisms were centrifuged, and resuspended in distilled water. This process was repeated four

<table>
<thead>
<tr>
<th>Concentration of egg albumin</th>
<th>Mm. per hr.</th>
<th>Potential difference</th>
<th>Agglutination after time noted at 20°C.</th>
</tr>
</thead>
<tbody>
<tr>
<td>per cent</td>
<td></td>
<td>millivolts</td>
<td>0.5 hr.</td>
</tr>
<tr>
<td>0</td>
<td>-7.5</td>
<td>-34.0</td>
<td>-</td>
</tr>
<tr>
<td>0.0003</td>
<td>-6.0</td>
<td>-27.0</td>
<td>-</td>
</tr>
<tr>
<td>0.001</td>
<td>-4.0</td>
<td>-18.0</td>
<td>-</td>
</tr>
<tr>
<td>0.003</td>
<td>-2.0</td>
<td>-9.0</td>
<td>-</td>
</tr>
<tr>
<td>0.010</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>0.03</td>
<td>+0.8</td>
<td>+3.4</td>
<td>-</td>
</tr>
<tr>
<td>0.10</td>
<td>2.0</td>
<td>+9.0</td>
<td>-</td>
</tr>
<tr>
<td>0.30</td>
<td>2.5</td>
<td>+11.2</td>
<td>-</td>
</tr>
<tr>
<td>0.90</td>
<td>3.2</td>
<td>+14.4</td>
<td>-</td>
</tr>
</tbody>
</table>

+ = agglutination visible with lens (8 diameters).
++ = agglutination visible without lens.
C. = complete settling, supernatant clear.

\textsuperscript{12} De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 395. See also Putter. 7
times. The sediment finally obtained was then suspended in a volume of distilled water equal to one-half that of the original broth. For the determinations one volume of this "standard" suspension was added to one or two volumes of the other solutions used. Table II shows that no noticeable change could be detected after the second washing.

**Effect of the Manner of Mixing and Time of Standing on the Potential.**—
No difference could be detected in the results obtained when the suspension was added to the solution or *vice versa*, provided the mixing was rapid and complete. As a rule the suspension was squirted into the solution from a pipette and mixed as thoroughly and rapidly as possible. No significant changes occurred in the potential measurements over an interval of 2 days except in the case of silver salts. The effect on the potential is, therefore, almost instantaneous in most cases. This is also true of the effect of immune serum, and shows that the time element consists in the time required for the organisms to come into contact. 13

**TABLE II.**

**Effect of Washing on Rate of Migration of Type D Suspension.**

100 cc. of broth culture, Type D, were centrifuged, suspended in distilled H₂O, centrifuged, and the process was repeated as noted. Migration was determined as noted.

<table>
<thead>
<tr>
<th>No. of times washed</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potential at pH 4.4</td>
<td>-9.0</td>
<td>-18.0</td>
<td>-27.0</td>
<td>-28.0</td>
<td>-28.0</td>
</tr>
<tr>
<td>&quot;  pH 3.0</td>
<td>-13.0</td>
<td>+1.6</td>
<td>+1.6</td>
<td>+1.6</td>
<td>+1.8</td>
</tr>
</tbody>
</table>

In the case of suspensions treated with silver salts at a pH of 4 or more, the potential drops rapidly and is very much lower after 24 hours. At the same time the suspension turns black so that the effect is probably due to the reduction of the silver.

**EXPERIMENTAL RESULTS.**

The results of the experiments are shown graphically in Figs. 2 to 8. The calculated potential in millivolts between the surface of the organism and the surrounding liquid is plotted as ordinates, and the salt concentration as abscissae. Since there is some doubt as to the correctness of the formula connecting velocities to millivolts, the actual velocities corrected for a potential drop of 1 volt per centimeter have also been given. The degree of agglutination is indicated by the character of the line. In the experiments in which no pH value

---

13 This conclusion had been reached by F. L. Gates (J. Exp. Med., 1922, xxxv, 63) in a study of the time required for adsorption of immune body.
Fig. 2. Effect of salts on the potential and agglutination of *B. typhosus*.

Fig. 3. Effect of acids on the potential and agglutination of *B. typhosus*.
Effect of salts on the potential and agglutination of the bacillus of rabbit septicemia Type D strain.

**Fig. 4.** Effect of salts on the potential and agglutination of *B. typhosus* at pH 2 (0.01 N HCl).

**Fig. 5.** Effect of salts on the potential and agglutination of the bacillus of rabbit septicemia Type D strain.
Fig. 6. Comparison of the acid agglutination of Type D and Type G strains of the bacillus of rabbit septicemia and the effect of immune serum and peptone on the potential and agglutination of Type G.

Fig. 7. Effect of salts on the potential and agglutination of Type D at pH 3 (0.001 N HCl).
is given, the pH was not regulated and the results are due in part to changes in the hydrogen ion concentration.

Inspection of the charts shows that in all experiments there is complete agglutination as soon as the potential is reduced below a value of about 15 millivolts (either positive or negative) provided the salt concentration is below 0.001 N. Below this salt concentration, therefore, the agglutination is seen to depend solely on the potential. Any substance which reduces the potential below about 15 millivolts will cause agglutination. There is another range of salt concentration above 0.10 N in which no agglutination occurs, although there is no measurable potential. Between these two ranges of salt concentration there is a zone in which agglutination occurs at various potential levels. This is evidently the result that we would expect if the salt acted in low concentration primarily on the potential, and in high concentration on the cohesive force. There would be an intermediate zone in which the agglutination could not be predicted from either measurement alone. This explanation is borne out by the measurements of the cohesive force shown in Fig. 9. These show that the cohesive force is markedly decreased in concentrations of more than 0.01 N; i.e., the range in which the critical potential begins to decrease. The figure shows that the effect on the cohesion is not connected with the valency nor with the electrical effects of the ions. LaCl₃ is far more effective than NaCl in reducing the potential, but

---

**Fig. 8.** Effect of salts on the potential and agglutination of Type D at pH 2.0.
less effective in reducing the cohesive force. The agglutination depends on both factors. It is possible, therefore, for all monovalent ions to affect the potential in the same way but to differ in their coagulating power. In order to predict the coagulating efficiency of a salt, it is therefore necessary to know the effect on both the potential and cohesion.

![Diagram](image-url)

**Fig. 9.** Effect of salts and acids on the cohesive force between films of *B. typhosus*.

The HCl curve differs from the others in that the cohesion is increased in solutions of higher concentration than 0.3 N. This agrees with the agglutination test (Fig. 3) which shows a zone of agglutination at this concentration.

The experiments show the result usually obtained in such cases, that low concentrations of salt precipitate and higher concentrations stabilize again. They also show that this is due in most cases to the
fact that excess salt or acid confers a high potential upon the particles, of opposite sign to that in low concentration.

These various effects are all shown in the case of thorium chloride (Fig. 2). In concentration below $5 \times 10^{-4}$ N no agglutination takes place since the potential is greater than 15 millivolts (the organisms being negative to the water) and the organisms are kept apart by the repulsion due to this potential. In concentrations between $5 \times 10^{-5}$ and $5 \times 10^{-4}$ N there is agglutination, since in this range the potential is less than 15 millivolts and the repulsion is therefore not sufficient to overcome the cohesion. In concentrations of from $5 \times 10^{-5}$ to $5 \times 10^{-1}$ the potential is greater than 15 millivolts (though of the opposite sign) and the suspension is again stable. At a concentration above 0.05 N the potential drops below 15 millivolts but agglutination does not occur since the cohesive force has also been reduced. A smaller potential is therefore sufficient to prevent agglutination. At a concentration of 0.10 N the potential is reduced practically to zero and agglutination again occurs. In still higher concentration the organisms are again stable due to a further decrease in the cohesive force. The hydrochloric acid curve is interesting in that it shows a zone of agglutination in concentrated solutions (> 0.3 N). This is due to the sudden increase in the cohesive force at this point as is shown in Fig. 9. This does not occur with the other chlorides and in the latter solutions no agglutination occurs in this range.

The stabilizing effect of sodium chloride in high concentration is shown more strikingly in Fig. 10, which gives the result of adding increasing salt on the acid agglutination zone. The addition of

14 According to O. Porges (Centr. Bath., 1 te Abt., Orig., 1906, xl, 133) agglutination occurs again in very strong salt solutions such as half saturated (NH$_4$)$_2$SO$_4$. This is probably a salting out phenomenon, due to a decrease in the forces between the surface of the particle and the liquid. For a review of the effect of salts on agglutination see Buchanan, R. E., J. Bact., 1919, iv, 82. The experiment itself shows that this is a different phenomenon since in saturated (NH$_4$)$_2$SO$_4$ agglutination occurs immediately whereas the type of agglutination studied in this paper requires considerable time.

15 It will be noted that in this experiment the isoelectric point was about pH 4.2 while in others with B. typhosus (Fig. 3) it is about 3.5. This difference was noted several times and depends probably on the age and condition of the suspension.
0.01 N salt decreases the potential and broadens the agglutination zone slightly. More concentrated salts, however, although it reduces the potential still more, decreases the agglutination, since the cohesive force is now being reduced. In concentrations of more than 1.0 N no agglutination occurs. The salt also shifts the zone of agglutination to the acid side. This result has been obtained by Michaelis and Rona with proteins.

Fig. 10. Effect of NaCl concentration on the potential and agglutination of B. typhosus at the acid agglutination zone.

Effect of the Salts on the Potential.—The experiments show the familiar result that the effect is due to the oppositely charged ion and increases in general with the valence of the ion. The effect is not purely due to the valence since the hydrogen ion is far more active than the other monovalent ions. The result also depends on the nature of the

suspension since the charge on the bacillus of rabbit septicemia may be reversed by sulfate or NaCl while with *Bacillus typhosus* suspension the charge is reduced but does not change in sign.

The experiments in Fig. 6 show clearly the reason for the characteristic difference in the stability of Types D and G of the rabbit septicemia bacillus. Type D which is very stable has a high potential whereas the potential of Type G is very little more than the critical.

The same figure shows that the acid agglutination zone may be shifted markedly by the addition of other substances. Peptone for instance moves it far to the acid side (cf. Putter) while immune body brings the isoelectric point to nearly 5. This point will be discussed more fully in the succeeding paper.

**Origin of the Potential.**—Loeb has shown, in the case of a protein solution separated from a solution of electrolyte by a collodion membrane, that the charge on the protein solution can be quantitatively accounted for on the basis of Donnan's theory of membrane potentials. According to this theory, electrolytes affect the potential of a particle in two ways. (1) By combining chemically with the particle (for example hydrogen ions). The ion then becomes part of the molecule of which the particle (membrane) is composed. As a result the concentration of this ion differs on the opposite sides of the membrane and gives rise to a potential. This potential may be calculated by Nernst's formula from the concentration of the common ion on both sides of the membrane. The membrane behaves as a reversible electrode for this ion. (2) Ions which affect the distribution of the common ion without further chemical combination with the membrane. This mechanism will suffice to account for all the observations made in the course of this work, if it be supposed that other ions than the hydrogen ion may act by chemical combination. The experiments are more complicated than those with a collodion membrane since the organisms are apparently more or less impermeable to ions.


18 Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 164, 165; *J. Gen. Physiol.*, 1921-22, iv, 463; also two papers in this number of the *Journal* which the writer has had the privilege of reading in manuscript form (*J. Gen. Physiol.*, 1921-22, iv, 741, 759).

SUMMARY.

1. Measurements have been made of the potential and of the cohesive force at the surface of *Bacillus typhosus* and the bacillus of rabbit septicemia in solutions of various salts and acids.

2. Electrolytes in low concentration (0.01 N) affect primarily the potential, and in high concentration decrease the cohesive force.

3. As long as the cohesive force is not affected, agglutination occurs whenever the potential is reduced below about 15 millivolts.

4. When the cohesive force is decreased the critical potential is also decreased, and in concentrated salt solution no agglutination occurs even though there is no measurable potential.