THE FLOCCULATION OF BACTERIA BY PROTEINS.

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The effect of the addition of proteins on the stability of suspensoid colloids has been the subject of numerous investigations. Ordinarily the proteins act to increase the stability of the suspension, hence their use as protective colloids. Under certain circumstances, however, they have been found to produce flocculation instead of protection. Neisser and Friedemann (1904) found that NaCl in a dilution incapable of producing flocculation by itself can coagulate mastic sol if one part of gelatin per million is present; blood serum, leech extract, and bacterial extract behaved in the same manner. Walpole (1913) reported that gelatin, albumin, and globulin, when added in high dilutions to oil emulsions or gold or mastic sols increased their sensitiveness to flocculation both by acids and salts. Brossa and Freundlich (1914) showed that dialyzed serum albumin, when added to $Fe(OH)_3$ sol, diminished the positive charge on the colloidal Fe $(OH)_3$ and caused it to be flocculated by concentrations of salt that were without effect upon the pure sol.

In the present investigation, the effect of pure proteins on the stability of bacterial suspensions at different H ion concentrations was studied. Several species of bacteria were used, but the most instructive results were obtained with a strain of *Bacterium coli*. This is because the point of optimum flocculation (the isoelectric point) for this organism lies in a very acid range, which leaves a wide interval between it and the isoelectric points of the different proteins studied. According to Beniasch (1912), *Bacterium coli* is not agglutinable by H ions. We have not found any recently isolated strains that would agglutinate in any of the buffer mixtures used by Beniasch; but most, though not all, strains of this organism will agglutinate in dilute acids in the absence of salt, at reactions ranging from pH 1.6 to 3.0. Putter

(1921) has also found *Bacterium coli* to agglutinate with acids in the absence of salt. The strain with which most of our work was done underwent a curious mutation about 2 months after it was first isolated. Our records show that on December 11 no agglutination took place in any of the acetate or lactate buffer mixtures; 3 days later the organism, with all its other characteristics unchanged, was found to agglutinate at pH 3.2 (negative at 3.0 and 3.5), both in the acetate and lactate buffer mixtures, but not in the phthalate buffers of Clark and Lubs (1917). No further change has been observed to the present time.

In all of these experiments, *Bacterium coli* was grown on beef extract peptone agar plates. The growth was suspended in 0.85 per cent NaCl, filtered through paper, centrifugated, then centrifugated three times out of distilled water. In most of the experiments, a temperature of 40°C. was employed. Flocculation was observed macroscopically; no test was considered positive unless definite macroscopic flocks were formed, which settled out to leave a clear supernatant fluid.

Lactic Acid-Sodium Lactate Buffer Mixture.

0.1 N lactic acid, *cc.*	0.6	1.2	2.4	5.0	10.0				
N lactic acid, *cc.*						2.0	4.0	8.0	16.0
Water, *cc.*	14.4	13.8	12.6	10.0	5.0	13.0	11.0	7.0	0
0.1 N sodium lactate, *cc.*	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
pH	4.7	4.4	4.1	3.8	3.5	3.3	3.0	2.7	2.4

Acetic Acid-Sodium Acetate Buffer Mixtures.

0.1 N acetic acid, *cc.*	0.6	1.2	2.4	5.0	10.0				
N acetic acid, *cc.*						2.0	4.0	8.0	16.0
Water, *cc.*	14.4	13.8	12.6	10.0	5.0	13.0	11.0	7.0	0
0.1 N sodium acetate, *cc.*	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
pH	5.6	5.3	5.0	4.7	4.4	4.1	3.8	3.5	3.2

Phosphate Buffer Mixtures.

0.1 N potassium dihydrogen phosphate, *cc.*	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
0.1 N sodium hydroxide, *cc.*	0.37	0.57	0.86	1.26	1.78	2.36	2.96	3.50
Water, *cc.*	14.63	14.43	14.14	13.74	13.22	12.64	12.04	11.50
pH	5.8	6.0	6.2	6.4	6.6	6.8	7.0	7.2

Lactic acid-lactate and acetic acid-acetate buffer mixtures were made up according to the method of Beniasch (1912), which gives mixtures of uniform salt concentration (0.025 M). This uniformity of salt concentration is essential, as the salt effect is very high in some cases. The phosphate buffer mixtures were made up according to the directions of Clark and Lubs (1917), except that they are made twice as dilute, to make the salt concentration equal to that of the Beniasch buffer mixtures.

Flocculation of Bacteria with Gelatin.

Isoelectric gelatin was prepared by the method of Loeb (1919). Suspensions of *Bacterium coli* were incubated with varying dilutions of gelatin, both in buffer mixtures (Table I) and in the absence of salt (Table II).

TABLE I.

Bacterium coli Suspension with Gelatin.

1.0 cc. buffer mixture + 0.5 cc. gelatin solution + 0.5 cc. *coli* suspension.

Concentration of gelatin.	Acetate buffers.								Lactate buffers.		
0	−	−	−	−	−	−	−	+	−	−	−
1:4,000,000	−	−	−	−	−	−	−	+	−	−	−
1:400,000	−	−	−	−	−	−	+	+	−	−	−
1:40,000	−	−	−	−	×	×	×	×	+	−	−
1:4,000	−	+	×	×	×	+	−	−	−	−	−
1:400	−	−	+	−	−	−	−	−	−	−	−
pH	5.3	5.0	4.7	4.4	4.1	3.8	3.5	3.2	3.0	2.7	2.4

Temperature = 40°C. × = agglutination within 30 minutes. + = agglutination within 6 hours.

In Table I it is to be noticed that with the highest concentration of gelatin, agglutination occurs only at pH 4.7, the isoelectric point of the gelatin. In the 1:4,000 dilution, the zone of flocculation widens, especially on the acid side, but it also includes the pH 5.0 tube. Beginning with the 1:40,000 dilution of gelatin, the zone of flocculation shifts over to the acid side, even becoming more acid than the flocculation zone of the control. It will be noted that there is an absence of flocculation with the higher concentrations of gelatin in the more acid tubes, forming a so called "proagglutinoid zone."

TABLE II.

Bacterium coli Suspension with Gelatin.

Acid and water to make 1.0 cc. + 0.5 cc. gelatin solution + 0.5 cc. *coli* suspension.

Concentration of gelatin.	0.002 N HCl.							0.02 N HCl.			0.2 N HCl.		
	0	0.05 cc.	0.1 cc.	0.2 cc.	0.3 cc.	0.5 cc.	1.0 cc.	0.2 cc.	0.5 cc.	1.0 cc.	0.2 cc.	0.5 cc.	1.0 cc.
0	—	—	—	—	—	—	×(3.0)	×	×	×	×(1.6)	—	—
1:4,000,000	—	—	—	—	—	—	×	×	×	×	×	—	—
1:400,000	—	—	—	—	—	×(3.4)	×	×	×	×	×	—	—
1:40,000	—	—	—	—	—	×	×	×	×	×	×	×(1.3)	—
1:4,000	—	—	−(5.2)	×(4.6)	×(4.0)	—	—	—	—	—	—	—	—
1:400	—	—	×(5.0)	×(4.6)	−(4.2)	—	—	—	—	—	—	—	—

Temperature = 40°C. Flocculation complete in 30 minutes. Reactions were determined colorimetrically as soon as flocculation was evident; the figures in parentheses indicate the pH.

Cataphoresis experiments with a micro apparatus similar to that of Putter (1921) show that the bacteria in this zone carry a positive charge.

In Table II, the experiment is repeated in the absence of salt. The general results are similar to those found in Table I, except that the zone of flocculation of untreated *Bacterium coli* is found to lie between pH 1.6 and 3.0. It will be noted that when isoelectric gelatin is added to a suspension of *Bacterium coli*, the mixture is more alkaline than pH 4.7; this is because the bacterial suspension, though carefully washed and in pure water, maintains a pH of 6.8 to 7.0 even though unprotected from the CO_2 of the air.

The use of indicators in determining the H ion concentration of unbuffered or poorly buffered solutions calls for a word of explanation. Tizard (1910) has shown with methyl orange and methyl red that serious errors can be made. In a series of unpublished experiments made in collaboration with C. B. Coulter, in which all results were checked electrometrically, it was found that a carefully purified methyl red indicator could be used without serious error in ranges from pH 4.6 to 5.4. With the various sulfonephthalein indicators, which can be made up in aqueous solution, it was found that if the dye be adjusted so that its pH is not far from the pH of the solution tested, quite reliable results could be obtained. Thus, brom thymol blue indicator, adjusted so that when viewed in thin layers it matched the pH 6.8 standard, can be used over the range pH 6.4 to 7.2, without an error greater than 0.2 pH. Without adjustment of the indicator for particular ranges, the error may be much greater than this.

The results obtained in these experiments are strikingly similar to those found by Michaelis and Davidsohn (1912) in the precipitation of nucleic acid (isoelectric at about pH 0.7) by serum albumin, which is isoelectric at pH 4.8. Maintaining a constant concentration of nucleic acid, these authors found a concentration of albumin that produced a precipitate whose optimum occurred at pH 4.1 to 4.4. On lowering the concentration of albumin, the optimum now shifted to the acid side, and the optimal zone was greatly broadened. This is essentially like what we have described in Tables I and II. Michaelis and Davidsohn explained their results on purely electrical grounds. Nucleic acid is negatively charged at all reactions alkaline to pH

0.7; albumin is positively charged at all reactions acid to pH 4.8. At all intermediate reactions, the two colloids are oppositely charged; they tend to attract each other and neutralize their charges; when this occurs, their combination is isoelectric and flocks out. Moreover, at the acid end of this intermediate zone, the nucleic acid particle is weakly, whereas the albumin is strongly charged, hence a smaller amount of albumin will be needed to combine with and discharge the nucleic acid than at the other end of the zone, where the strongly charged nucleic acid would require larger amounts of the more weakly charged albumin to neutralize it. This view is capable of application to such phenomena as the flocculation of *Bacterium coli* by gelatin at pH 5.0 and 3.0 (Table I), points that lie outside of the intermediate zone. As these authors point out in another paper (1913), any solution of ampholyte at every pH exists in three forms,—as anion, as cation, and as undissociated molecule; it is the relative concentration of these three species that changes with H ion concentration. Cationic gelatin exists, therefore, at pH 5.0; anionic gelatin exists at pH 3.0. At pH 5.0, one might conceive that the bacterial cell (which is here negatively charged) would unite with the small amount of gelatin cations present; the gelatin cations being thus removed from solution, more would be liberated, this process continuing until a state of equilibrium between gelatin cations in solution and gelatin cations combined was reached. If the charge on the cell-gelatin complex is below a certain threshhold value when equilibrium is reached, agglutination may occur, otherwise not. As it is the pH that determines the amount of gelatin cations in solution, it likewise determines the amount that can be combined when equilibrium is reached; hence it can be understood why agglutination, though occurring at pH 5.0, might not occur at pH 5.3. The same explanation would apply to the flocculation of *Bacterium coli* by proteins at reactions more acid than the flocculation zone of the untreated bacteria.

It is more difficult to apply this theory to the pro-zone observed in these experiments. Thus (Table I) when the gelatin concentration is 1:400, no agglutination of the bacteria occurs at reactions from pH 4.4 to 3.0, though more dilute gelatin causes flocculation. The bacteria in this zone are charged positively. If a primary electrical neutralization occurred, it must have been followed by further com-

bination with gelatin cations, and this must have required a different mechanism. We cannot exclude the possibility that the primary cause of union between bacteria and protein is non-electrical (perhaps a surface tension phenomenon), and that electrical neutralization is a secondary effect which may or may not occur.

Flocculation with Crystallized Egg Albumin.

Crystallized egg albumin was obtained by the method of Hopkins and Pinkus (1898); this was dialyzed to remove the ammonium sulfate. Experiments were conducted in buffer mixtures only (Table III).

TABLE III.

Bacterium coli Suspension with Egg Albumin.

1.0 cc. buffer mixture + 0.5 cc. albumin solution + 0.5 cc. *coli* suspension.

Concentration of albumin.	Acetate buffer.	Lactate buffers.								
0	−	−	−	−	−	−	+	−	−	−
1:400,000	−	−	−	−	−	−	+	−	−	−
1:40,000	−	−	−	−	+	+	+	+	−	−
1:4,000	−	−	×	×	×	×	×	+	−	−
1:400	−	−	×	×	−	−	−	−	−	−
pH	5.0	4.7	4.4	4.1	3.8	3.5	3.3	3.0	2.7	2.4

Temperature = 40°C. × = agglutination within 1 hour. + = agglutination within 4 hours.

Agglutination of this strain of *Bacterium coli* did not occur at pH 4.8, the isoelectric point of the albumin. A second strain of this organism was agglutinated at pH 4.7 by an albumin concentration of 1:150.

Flocculation with Protalbumose and Heteroalbumose.

These albumoses were prepared from Witte's peptone by the method of Pick (1898). The heteroalbumoses gave a clear solution when boiled and adjusted to pH 7.0; the protalbumose required no adjustment to give a clear solution.

The protalbumose was completely soluble at all reactions tested. The heteroalbumose, in a concentration of 1:400, was turbid at reac-

tions between pH 4.4 and 6.4, with a maximum turbidity at pH 5.3 and 5.6.

TABLE IV.

Bacterium coli Suspension with Protalbumose.

1.0 cc. buffer mixture + 0.5 cc. protalbumose solution + 0.5 cc. *coli* suspension.

Concentration of protalbumose.	Lactate buffers.								
0	−	−	−	−	−	+	−	−	−
1:4,000,000	−	−	−	−	×	×	−	−	−
1:400,000	−	−	−	−	×	×	×	×	−
1:40,000	−	−	−	−	×	×	×	×	−
1:4,000	−	−	−	×	×	×	×	−	−
1:400	−	×	×	×	×	×	×	−	−
pH	4.7	4.4	4.1	3.8	3.5	3.3	3.0	2.7	2.4

Temperature = 40°C. All agglutinations were complete in 1 hour.

TABLE V.

Bacterium coli Suspension with Heteroalbumose.

1.0 cc. buffer mixture + 0.5 cc. heteroalbumose solution + 0.5 cc. *coli* suspension.

Concentration of heteroalbumose.	Phosphate buffers.			Acetate buffers.									Lactate buffers.		
0	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
1:4,000,000	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
1:400,000	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−
1:40,000	−	−	−	−	−	−	−	−	+	×	×	×	×	×	−
1:4,000	−	−	−	−	−	×	×	×	×	×	×	×	−	−	−
1:400	−	+	×	×	×	×	×	×	×	×	+	−	−	−	−
pH	6.2	6.0	5.8	5.6	5.3	5.0	4.7	4.4	4.1	3.8	3.5	3.2	3.0	2.7	2.4

Temperature = 40°C. × = agglutination within 30 minutes. + = agglutination within 3 hours.

Flocculation with Edestin.

Crystalline edestin was prepared from hemp seed by the method of Osborne (1901). This substance was found to be practically insoluble in water or in the buffer mixtures used at reactions between pH 5.6 to 9.6. Rona and Michaelis (1910) report pH 6.9 as the

isoelectric point of edestin, though in a later paper Michaelis and Mendelssohn (1914) give it a value of pH 5.6.

In the experiment shown in Table VI, a 0.25 per cent suspension of edestin in distilled water was dissolved by the addition of minimal alkali; the solution was immediately distributed in the buffer mixtures and incubated at 40°C. for 30 minutes. The 1:1,600 dilution of edestin gave a small precipitate in the pH 5.3 tube, and a heavier flocculent precipitate in the tubes alkaline to this. *Bacterium coli* suspension was then added without stirring up the precipitate that had formed.

TABLE VI.

Bacterium coli Suspension with Edestin.

1.0 cc. buffer mixture + 0.5 cc. edestin solution + 0.5 cc. *coli* suspension.

Concentration of edestin.	Phosphate buffers.		Acetate buffers.								
0	−	−	−	−	−	−	−	−	−	−	+
1:1,600,000	−	−	−	−	−	−	−	−	−	−	+
1:160,000	−	−	−	−	−	−	−	−	+	×	×
1:16,000	−	−	−	×	×	×	×	×	×	+	−
1:1,600	−	+	×	−	−	−	−	−	−	−	−
pH	6.0	5.8	5.6	5.3	5.0	4.7	4.4	4.1	3.8	3.5	3.2

Temperature = 40°C. × = agglutination within 30 minutes. + = agglutination within 6 hours.

Flocculation with Hemoglobin.

Crystallized oxyhemoglobin was prepared from the blood of the horse, the dog, and the guinea pig, some by the method of Hoppe-Seyler (1903), and some by the method of Dudley and Evans (1921). No marked differences in behavior were observed between these crystallized oxyhemoglobins and other solutions prepared by laking washed erythrocytes and centrifugating out the stroma. In the results obtained, there were some differences between the hemoglobins of different species of animals, and between the oxy- and methemoglobins of the same species.

It will be observed that in the most concentrated hemoglobin that we used (Table VII) the zone of flocculation extended only as far toward the alkaline side as pH 6.2 (in some experiments,

to pH 6.4). As we have observed that when we added a 1 per cent pure oxyhemoglobin solution to two or three volumes of *Bacterium coli* suspension, flocculation occurred without any adjustment of reaction, we suspected that salts interfered with the agglutination near the isoelectric point of the hemoglobin. In one experiment, where H ion concentrations were determined electrometrically, flocculation of the bacteria occurred in the absence of salt at pH 6.71 and points acid to this, the concentration of hemoglobin being 1:400.

It is also to be noted in Table VII that there is no pro-zone with the higher concentrations of hemoglobin, such as we have uniformly

TABLE VII.

Bacterium coli Suspension with Guinea Pig Oxyhemoglobin.
1.0 cc. buffer mixture + 0.5 cc. oxyhemoglobin solution + 0.5 cc. *coli* suspension.

Concentration of hemoglobin.	Phosphate buffers.				Acetate buffers.				
0	−	−	−	−	−	−	−	−	−
1:250,000	−	−	−	−	−	−	−	−	−
1:50,000	−	−	−	−	−	−	×	×	×
1:10,000	−	−	−	−	×	×	×	×	×
1:20,000	−	+	×	×	×	×	×	×	×
1:400	−	+	×	×	×	×	×	×	×
pH	6.4	6.2	6.0	5.8	5.6	5.3	5.0	4.7	4.4

Temperature = 20°C. × = agglutination within 1 hour. + = agglutination within 12 hours.

found with the other proteins studied. We did not extend the series more acid than pH 4.4, because the greenish brown color of the solutions showed that we no longer had oxyhemoglobin. When *Diplococcus pneumoniæ* suspensions were used instead of *Bacterium coli*, a pro-zone was obtained; when the hemoglobin concentration was 1:400, the bacteria were charged positively at pH 5.0 and reactions acid to this, and remained non-agglutinated. When, instead of bacteria, we used an aqueous suspension of cellulose nitrate as substrate, hemoglobin in dilutions of 1:2,000 to 1:10,000 caused agglutination of the cellulose nitrate at reactions between pH 6.2 to 7.0 (in the absence of salt); with higher dilutions of hemoglobin, flocculation took place at more acid reactions.

The experiments described above establish the influence of proteins upon the stability of bacterial suspensions at different H ion concentrations, and relate this influence to the isoelectric point of the added protein and its concentration. It must be noted that bacteria are not the only suspensions that are effected by proteins in this way. We have tested the effect of proteins on sols of cellulose nitrate, cellulose acetate, and paraffin emulsions, with results that closely parallel our experiments with bacteria. Although Walpole (1913) does not relate his results to the isoelectric point of the protein, it seems clear, from the curve given by him, that gelatin, albumin, and globulin, affect the stability of oil emulsions and mastic and gold sols at different concentrations of H ions in the same manner that these substances affect bacterial suspensions. The same may be said of the action of albumin on $Fe(OH)_3$ sol at different salt concentrations, as reported by Brossa and Freundlich (1914). Of the same import are the observations of Putter (1921) that in the presence of peptone, acids change the sign of the charge on *Bacterium coli*, and of Coulter (1922) who shows that erythrocytes in contact with specific sensitizer, or even with normal homologous or heterologous sera, agglutinate most promptly when the pH is such that the euglobulins of these sera are isoelectric.

Loeb (1920) has shown that when collodion membranes are treated with proteins, a combination takes place between the collodion and the protein, and the membrane becomes isoelectric near the H ion concentration at which the protein is isoelectric. Reactions acid to this point now charge the membrane positively. It is obvious that the effect of proteins upon bacteria (and probably upon other suspensions and emulsions) is of the same nature. Where concentrations of about 0.25 per cent of protein are used, the bacteria agglutinate at or near the isoelectric point of the protein; increasing the H ion concentration beyond this point causes the original charge upon the bacteria to be reversed, and prevents agglutination.

SUMMARY.

1. The effect of adding pure proteins to bacterial suspensions at different H ion concentrations has been studied.

2. The zone of flocculation of protein-treated bacteria bears a significant relationship to the isoelectric point of the protein used. With the higher concentration of protein, agglutination occurs at or near the isoelectric point of that protein; at reactions acid to this, the bacteria carry a positive charge and are not agglutinated. With diminishing concentration of protein, the zone of flocculation shifts toward and goes beyond that characteristic of the untreated bacteria. This occurs both in the presence and absence of salts.

3. A diversity of other suspensions, such as sols of gold, mastic, cellulose nitrate, cellulose acetate, $Fe(OH)_3$, oil emulsions, and erythrocytes, have been found by ourselves and others to exhibit a similar altered stability when treated with proteins in the same way.

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