FLOCCULATION OF BACTERIA BY HYDROPHILIC COLLOIDS

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Bacteria, like many colloids, are generally considered to be negatively charged at a neutral pH. In many cases, bacteria behave as typical hydrophilic colloids and are not precipitated nor flocculated by merely reducing the surface charges to zero (Lamanna and Mallette, 1953). When this is the situation, it is necessary to add dehydrating agents or specific antibodies to obtain flocculation. Observations on the flocculation of several bacterial species, in particular *Pasteurella tularensis*, by a number of hydrophilic colloids are presented in this report.

Successful use of starch, gelatin, and glue in the treatment of coal washery effluents has been described by Tomalin (1938), by Needham, (1936), and by Samuel (1936). In these processes the colloidal clay particles were flocculated by electrolytes, while the somewhat larger coal particles were flocculated by the organic additives. Study of the possible use of gelatin, pectin, and methylcellulose as blood plasma substitutes, led to the observation that these materials increased the sedimentation rate of erythrocytes when injected intravenously (Hueper, 1944). Addition of these materials (0.02 per cent concentration) to blood was suggested by Levine and Hoyt (1946) as a method to reduce the settling time of red blood cells from several days to five hours in the preparation of blood plasma. The ability of many polysaccharides to improve the physical structure of soil, by promoting aggregation of finely divided colloidal clay particles has been observed by several workers. Bartholomew and Norman (1941) noted a relationship between fertility and uronic acid content of soils. The beneficial effect of methylcellulose on soil was reported by Felber (1944). The improved structure, aerating ability, and water-holding capacity of soils observed after the addition of bacterial polysaccharides (Martin, 1946), alginic acid (Quastel and Webley, 1947), cellulose acetate, methylcellulose, and carboxymethylcellulose (Quastel, 1952), led to use of synthetic polyelectrolytes as soil conditioners (Hedrick and Mowry, 1952). Quartaroli (1952) noted that mucilaginous extracts of seeds, particularly cress seed, flocculated clays. Gelatin has been widely used as a clarifying agent in fruit juices and wines containing tannin. Gelatin in low concentrations reduces the amount of acid required to precipitate *Escherichia coli*, (Eggerth and Bellows, 1921–1922). Gelatin, at relatively high concentration, effectively flocculated *P. tularensis* and *Brucella suis* in neutral solutions (Morée, M. T., 1950, *unpublished data*). Avi-Dor and Yaniv (1953) noted that virulent strains of *P. tularensis* were not agglutinated by electrolytes or basic polyelectrolytes.

Since the completion of this study a series of reports on the flocculation and filtration of colloidal phosphate slimes have been published by La Mer and Smellie (1956*a*, *b*), Smellie and La Mer (1956), and La Mer *et al.* (1957). Many of the compounds that were effective for flocculation of the phosphate slimes we found effective for precipitating bacteria. However, with bacteria the flocculation is much slower and requires 5 to 10 times the concentration of flocculant.

EXPERIMENTAL METHODS

A virulent strain of *P. tularensis* was grown in a medium containing (in per cent): acid-hydrolyzed casein, 1; yeast extract, 0.5; glucose, 2.0; cysteine HCl, 0.01; thiamin, 0.0001; NaCl, 0.5; KH_2PO_4, 0.28; and K_2HPO_4, 0.12. *Serratia marcescens* was grown in a medium of the same composition. Cultures were incubated in shake-flasks for 16 hr at 37 C. *P. tularensis* in this medium produced 20 to 30 $\times$ 10^9 cells per ml. Cultures of *Escherichia freundii* and *Proteus morganii* were grown for 16 hr in shake-flasks at 30 C, in a medium consisting of 1.0 per cent Tryptose (Difco) and 0.5 per cent glucose. All cultures were neutralized to pH 7.0 with N NaOH or N H_2SO_4 prior to use in flocculation experiments.

Stock solutions of the colloids tested in this study were prepared in from 1 to 20 per cent concentrations depending on the colloid. The dry powders were added to distilled water gradu-

ally and with agitation until solution was complete. Materials occurring naturally were autoclaved or heated to 80 C for 10 min. The stock solutions were held at 4 C until used.

In performing flocculation experiments, aliquots of cultures were added to various quantities of each colloid in test tubes, to give a total volume of 20 ml. The tubes were then stoppered and the colloid and culture thoroughly mixed by inverting the tubes approximately 20 times. One 10-ml aliquot was dispensed into a 10-ml graduated test tube for determination of final precipitate volume. The degree of cell concentration achieved (indicated by the term "concentration factor") was obtained by dividing the volume of culture by the final precipitate volume. The second 10-ml portion was placed in a standardized Coleman turbidity tube and the flocculation followed by observation of per cent light transmittance with a Coleman model 14 spectrophotometer. The spectrophotometer was set at 100 per cent light transmittance using uninoculated medium as a standard at a wave length of 650 mμ. Unless otherwise stated, the tubes were held at 37 C in a water bath for 8 hr, then at 4 C for the duration of each experiment.

For quantitative recovery data, the culture supernatant was decanted and plate counts made after appropriate dilution in sterile diluent containing 0.1 per cent Tryptose plus 0.5 per cent sodium chloride. The precipitate was brought up to the original culture volume with sterile diluent, the cells redispersed by gentle agitation, then serially diluted and plated. *P. tularensis* was plated on glucose-cysteine-blood-agar (Downs *et al.*, 1947) by a surface smear technique. The other species were plated on nutrient agar.

RESULTS

Table 1 presents data on the settling rates, concentration, and recovery obtained with *P. tularensis*, using the twelve most effective colloids tested. These data represent average values from two replicate experiments in which the colloid concentrations were varied so that a bracketing of the optimum concentration was obtained for each colloid in each experiment. When suboptimum or excess colloid was added, a reduced settling rate was observed. It will be noted that all of the materials produced essentially complete flocculation and that viable cell recoveries above 90 per cent were obtained after redispersion of the precipitate. In most instances, 30- to 40-fold concentrations were obtained. With the exception of gelatin, the optimum colloid concentration for flocculation was within the range of 0.15 to 0.30 per cent. Methylcellulose, carboxymethylcellulose (sodium salt), propylene glycol alginate, and pectin produced the most rapid flocculation. These flocculants contained a high proportion of hydroxyl- or carboxyl-groups. The commercial flocculating agents Lytron X-886, DX-908, and

TABLE 1

Flocculation of Pasteurella tularensis by hydrophilic colloids

Colloid		Turbidity		Concentration factor	Recovery, 18 hr	
Per cent in stock solution	Optimum per cent for flocculation	Per cent light transmittance after settling for indicated time, hr			Per cent original cells in	
		2	18	18 hr	Supernatant	Precipitate
Methylcellulose (400) 2.0%	0.20	65	95	30.0	0.76	94.0
Carboxymethylcellulose(MV) 2.0%	0.27	49	89	36.0	1.10	105.0
Hydroxyethylcellulose, WP-40 2.0%	0.26	26	72	34.0	2.90	93.0
Sodium alginate, 1.5%	0.19	35	89	28.0	1.10	93.0
Propyleneglycol alginate, 1.0%	0.21	58	86	34.5	0.63	98.0
Pectin (citrus) 4.0%	0.20	48	88	35	0.51	93.0
Locust bean gum 1.5%	0.15	47	83	36	0.92	91.0
Gum guar 1.0%	0.19	40	84	26	0.56	109.0
Gelatin (USP) 20%	4.00	23	81	12	1.80	92.7
Lytron-X886 1.0%	0.3	39	89	28	0.86	98.9
DX-908 1.0%	0.15	30	86	33	1.75	94.0
Separan 2610, 1.0%	0.27	18	80	34	1.30	103.0
Control	—	4	4			

Separan 2610 are high molecular weight linear polymers with carboxyl- or amide-groups available for adsorption on particles. In all cases, the cells were easily redispersed and remained in suspension after dilution of the precipitates to the original culture volume.

Tables 2, 3, and 4 show similar data on the flocculation of *E. freundii*, *S. marcescens*, and *P.*

TABLE 2

Flocculation of Escherichia freundii by hydrophilic colloids

Colloid		Turbidity	Concentration Factor	Recovery, 18 hr
Per cent in stock solution	Optimum per cent for flocculation	Per cent light transmittance after settling for 18 hr	18 hr	Per cent original cells in supernatant
Socium alginate, 1.5%	0.15	59	18	1.7
Lytron-X886, 1.0%	0.20	65	16	1.6
Methylcellulose, 2.0%	0.40	77	40	0.1
Carboxymethylcellulose, 2.0%	0.10	60	24	1.1
Pectin, 4.0%	0.40	77	30	0.8
Control	—	7	—	(10 × 10^8 VCC)*

* Viable cell count of original culture.

TABLE 3

Flocculation of Serratia marcescens by hydrophilic colloids

Colloid		Turbidity	Concentration Factor	Recovery at 18 hr
Per cent in stock solution	Optimum per cent for flocculation	Per cent light transmittance after settling for 18 hr	18 hr	Per cent original cells supernatant
Sodium alginate, 1.5%	0.15	38	18.0	1.7
Methylcellulose, 2.0%	0.20	60	18.0	3.0
Pectin, 4.0%	0.8	48	7.5	1.5
Gum guar, 1.0%	0.3	48	10.0	2.3
Locust bean gum, 1.5%	0.3	66	7.5	3.0
Control	—	3	—	(43.6 × 10^9 VCC)*

* Viable cell count of original culture.

TABLE 4

Flocculation of Proteus morganii by hydrophilic colloids

Colloid		Turbidity	Concentration Factor	Recovery at 18 hr
Stock solution	Optimum per cent for flocculation	Per cent light transmittance after settling for 18 hr	18 hr	Per cent original cells in supernatant
Sodium alginate 1.5%	0.15	60	45	5.0
Methylcellulose, 2.0%	0.40	56	40	1.0
Pectin, 4.0%	0.40	47	45	5.0
Gum guar, 1.0%	0.10	66	30	4.9
Locust bean gum, 1.5%	0.30	59	32	10.0
Control	—	10	—	(39.0 × 10^8 VCC)*

* Viable cell count of original culture.

morganii. It will be noted that the efficiency, the optimum concentration of the colloid, and the degree of concentration of cells achieved varied with each organism. Pectin and methylcellulose, at optimum concentrations which varied from 0.1 to 0.4 per cent, also were effective in flocculating *Micrococcus aureus*, *Sarcina lutea*, *Salmonella typhosa*, *Shigella paradysenteriae*, *Brucella suis*, vegetative cells of *Bacillus subtilis*, and spores of *Bacillus anthracis*.

A number of other materials were tested for ability to flocculate *P. tularensis*. Gums karaya, tragacanth, and ghatti, were effective at 0.2, 0.5, and 1.0 per cent concentrations, respectively. However, these colloids were also precipitated with the cells, forming three-layered mixtures. Carragheenates formed a voluminous, viscous floc. Starch, (tapioca, potato, or corn), starch derivatives (Polyoses, Corn Products Refining Co., N. Y.) dextrin, dextrans, sodium polypectate, polyvinylpyrrolidone, polyacrylates, and gums arabic and guaiac were ineffective.

Effect of heat-treatment and hydration of colloids. In preliminary experiments with the materials occurring naturally and methylcellulose, difficulty was experienced in duplicating results. This difficulty was traced to variations occurring in the preparation and storage of stock solutions. When dry sodium alginate, gum guar or locust bean gum were sterilized by carboxyclaving with ethylene oxide and carbon dioxide, and then dissolved in sterile water, the resulting solutions were inactive or not completely effective as flocculating agents. Heating pectin and sodium alginate solutions at 80 C for 10 min yielded optimum results, but the flocculating ability of these materials was completely destroyed by autoclaving. The ability of gum guar and locust bean gum solutions to cause rapid flocculation was enhanced by autoclaving at 121 C for 15 min.

The efficiency of methylcellulose as a flocculant was not affected by heating, but, as shown in table 5, this colloid required a period of low-temperature storage after preparation to be effective. Use immediately after preparation yielded negative results. It was thought that these conflicting results were probably caused by the degree of hydration of the methylcellulose used. An additional experiment was performed to determine the effect of hydration of methylcellulose on flocculation. It is well known that, in solution, methylcellulose can exist in various states of hydration, the degree of hydration being readily controlled either by heat treatment or by salt content of the solution (Methocel Handbook, 1953; Ott, 1943). Thus, methylcellulose is insoluble in hot water but dissolves readily at 37 C and becomes progressively more hydrated as the temperature is reduced. Also, sodium chloride, when present with methylcellulose in solution, competes with methylcellulose for water so that the degree of hydration decreases with an increase in salt concentration. Using these methods in combination, the following experiment was performed to determine the effect of hydration of methylcellulose on flocculation of *P. tularensis*.

TABLE 5

Settling rates of Pasteurella tularensis cells flocculated with 0.2 per cent methylcellulose after indicated treatment

Methylcellulose Sterilized by:			Per Cent Light Transmittance of Culture after Indicated Settling Time at 37 C					
Carboxy-claving*	Heat treatment		Stock solutions used immediately			Stock solutions held 16 hr at 4 C after preparation		
Time, hr	Time, min	Temp C	3 hr	5 hr	22 hr	3 hr	5 hr	22 hr
16	—	—	10	11	11	72	85	98
	60	121	11	10	11	71	78	100
	120	121	11	11	11	66	81	100

* Sterile H_2O added to colloid after sterilization.

Solutions were prepared so as to contain 2 per cent methylcellulose and the desired sodium chloride content (0 to 10 per cent). These solutions were shaken at 37 C for 72 hr to obtain equilibrium conditions. The aliquots were diluted 1:9 with a culture of *P. tularensis*. After these samples had been held at 37 C for 12 and 24 hr, they were examined turbidimetrically for precipitation of bacterial cells. In no sample had precipitation occurred. This observation indicated that at 37 C the methylcellulose had not hydrated sufficiently, regardless of the salt concentration. Consequently, it appeared that temperature was the critical factor in hydration of the colloid.

To further evaluate the conditions affecting hydration of methylcellulose, the original solutions of colloid and salt which had been prepared and shaken for 72 hr at 37 C were stored for 16 hr at 4 C. Aliquots were diluted 1:9 with culture and the resulting samples held at 37 C for 12 hr

TABLE 6

Flocculating properties of methylcellulose of various degrees of hydration

Per cent NaCl in 2% Stock Solution of Methylcellulose*	Per Cent Light Transmittance of *Pasteurella tularensis* Cultures Prepared by Diluting Stock Solutions 1:9 and Held as Indicated			
	hr at 37 C			12 hr at 37 C plus 12 hr at 4 C
	3	6	12	
0–2	55	76	82	89
3	48	67	75	84
4	38	58	73	82
5	16	21	22	76
6	15	17	18	80
7–10	15	16	16	74
Control culture alone	15	15	15	15

* Held at 37 C for 72 hr, then stored at 4 C for 16 hr.

and examined for precipitation (table 6). The results showed that precipitation of the bacterial cells occurred if the original salt concentration was 4 per cent or less, indicating that methylcellulose was sufficiently hydrated under these conditions. However, when the original salt concentration was 5 per cent or greater, it appeared that the salt had a greater affinity for water than had the colloid; thus, little hydration of methylcellulose occurred.

The tubes of culture, colloid, and salt used in the latter experiment were stored at 4 C for 12 additional hr and reexamined for precipitation. The results showed that in all of the samples the cells had precipitated. Thus, under the conditions of the experiment and with additional storage time at the reduced salt concentration, methylcellulose had hydrated to such a degree that it precipitated cells.

Results of subsequent experiments using colloid-sucrose-culture mixtures showed that the ability of both methylcellulose and pectin to cause flocculation was decreased progressively by increasing amounts of sucrose. At a 50 per cent sucrose concentration no flocculation occurred.

Effect of enzymatic hydrolysis of colloids on flocculation. To determine approximately the relationship of chain-length of the polymeric colloids under study to flocculation power, several of these materials were subjected to enzymatic digestion and the resulting products tested for their ability to flocculate cells of *P. tularensis.*

Enzymatic digestion of a 0.4 per cent pectin solution with 0.5 per cent Pectinol M (Rohm and Hass, Philadelphia), a commercial polygalacturonase, at 37 C for 15 min completely negated the flocculating ability of the material. Similar results were obtained when locust bean gum, methylcellulose or carboxymethylcellulose were hydrolyzed with Diatane-50, (George A. Jeffries and Company, Salem, Va.) an industrial fungal amylase, or when gelatin was trypsin-digested. In all cases when the treated colloids had been digested to lower molecular weight components, producing solutions of approximately 50 per cent of their initial viscosity, the flocculating ability had been destroyed.

DISCUSSION

It has been noted that flocculation of bacteria occurs with a variety of hydrophilic colloids. The precipitating agent may be a protein, a synthetic polymer, or a naturally occurring or modified polysaccharide containing glucose, galactose, or mannose derivatives as constituent carbohydrates. Most of these materials are negatively charged, but methylcellulose, for example, is an electrostatically neutral, nonionized compound, and gelatin is effective at, above, and below its isoelectric point. Flocculation is not influenced appreciably by a range in pH of 4.0 to 8.0. Although trace quantities of electrolytes are required, flocculation occurs over a wide range of electrolyte concentration. Many of the colloids can be degraded enzymatically or by heat and thereby rendered noneffective. It is evident, therefore, that this type of flocculation is not due to charge-neutralization of the bacteria.

All of the nonprotein flocculants have certain characteristics in common. They are long, linear, rigidly extended chain, hydrophilic molecules, capable of forming highly viscous solutions at low concentrations by molecular association (McBain, 1950; Stoloff, 1954). These characteristics and the high content of alcoholic groups of the polysaccharides suggest that the flocculation mechanism proposed by Ruehrwein and Ward (1952), Ruehrwein (1955), and Michaels (1954) for aggregation of clay particles by polyelectrolytes is applicable in this case. In the proposed mechanism, the colloids function by adsorbing on the hydrated surface of the particles and

bridging between the suspended particles with subsequent aggregation or flocculation. The adsorption occurs by hydrogen-bonding between hydroxyl, nonionized carboxyl, or amide groups of the polymers and the hydroxyl group of the particle. The high concentration of gelatin required for flocculation may be explained by the fact that a large proportion of gelatin molecules in solution are coiled to a small fraction of their extended length (Gouinlock, 1955), and, therefore, are ineffective in bridging.

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SUMMARY

A number of hydrophilic colloids flocculate *Pasteurella tularensis*, *Escherichia freundii*, *Serratia marcescens*, *Proteus morganii*, and other bacterial species in culture media at pH 7.0. Hydration of the flocculants is essential. Flocculating properties of these materials are negated by enzymatic digestion or, in some cases, by heat treatment. A mechanism previously advanced to explain the aggregating action of polyelectrolytes on clay suspensions, which involves bridging by linear molecules, appears applicable to this type of bacterial flocculation.

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