MECHANISM OF THE NON-SPECIFIC SERUM AGGLUTINATION OF THE OBLIGATE ANAEROBES CLOSTRIDIUM PARAPUTRIFICUM AND CLOSTRIDIUM CAPITOVALIS

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Non-specific reactions in agglutination tests have long provided trouble for the bacteriologist. One of the chief offenders has been spontaneous agglutination. The pseudo-reactions obtained in the serologic agglutination of the obligate anaerobes *Clostridium paraputrificum* (1934) and *Clostridium capitovalis* (1935), however, were not considered as due to spontaneous agglutination, but rather to the acid produced in the glucose broth cultures used. When the acid was eliminated it was easy to differentiate these species on the basis of agglutination.

At first it was thought that acid alone was responsible for the cross-agglutinations occurring when glucose broth cultures of these species were tested against heterologous serums. It was found, however, that a constituent of the broth was also a contributing factor. The relation of these serum-broth-acid reactions to the acid agglutinations described by Michaelis (1911) was also studied.

I. THE pH VALUES OF NON-SPECIFIC AGGLUTINATION WITH BROTH CULTURES OF CL. PARAPUTRIFICUM

The pH values at which non-specific agglutination occurred with broth cultures of *Cl. paraputrificum*, strain 4453, was roughly determined by using buffer solutions of pH 7.0, 6.0, 5.0, and 4.0 approximately. These were made according to McIlvaine's

tables (1921). Normal rabbit serum was then diluted from 1:10 to 1:2560 in each buffer solution in order to maintain the customary 2.0 cc. volume of the macroscopic agglutination test. These serum-buffer mixtures were allowed to stand at least one hour. No changes were observed in this time or after three hours incubation. Because *Cl. paraputrificum* at times failed to grow in peptone meat infusion broth heavily enough to provide satisfactory suspensions, the organisms were grown in glucose broth for forty-eight hours, twice centrifugalized and washed in distilled water, and re-suspended in peptone meat infusion broth. These suspensions were filtered through cotton and then added in equal quantities to the serum-buffer mixtures. Controls of the peptone meat infusion suspensions and of each buffer without serum were included in each test. At the same time a parallel series of glucose broth cultures was tested. The mixtures were incubated at 37°C. for three hours before the reactions were recorded and the hydrogen-ion concentrations determined colorimetrically. Controls of the bacterial suspensions and normal serum diluted in physiological saline were also tested. The two series are compared in table 1.

Table 1 shows that agglutination occurred in both series between pH 4.6 to 5.2. No flocculation occurred with the peptone infusion broth suspension in serum-saline at the pH of 6.8, but partial agglutination was found up to 1:80 in the serum-saline and glucose broth culture mixtures, the pH values of which were approximately 5.1. These reactions indicated that in a narrow pH zone serum in concentrations greater than 1:80 aided flocculation because any marked increase in hydrogen-ion concentration gave more or less complete agglutination in all tubes including the controls with no serum.

To determine as closely as possible the critical value of the reaction, potentiometric measurements were made with a glass electrode.[1] Cultures of *Cl. paraputrificum* were grown for forty-eight hours at 37°C. in glucose broth under "vas-par," twice centrifugalized and washed in distilled water, and resuspended in

[1] The author is indebted to Dr. Robert M. Hill of the Department of Biochemistry for these measurements.

TABLE 1

Zone of hydrogen-ion concentration giving non-specific agglutination of broth cultures of Cl. paraputrificum in the presence of normal serum

pH RANGE	SERUM DILUTIONS									NO SERUM
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	
A. Peptone meat infusion suspensions										
7.0	–	–	–	–	–	–	–	–	–	–
6.1	–	–	–	–	–	–	–	–	–	–
5.2	–	–	–	–	–	–	–	–	–	–
4.4–4.6	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
6.8*	–	–	–	–	–	–	–	–	–	–
B. Glucose broth cultures										
7.1	–	–	–	–	–	–	–	–	–	–
6.2	–	–	–	–	–	–	–	–	–	–
5.2	–	–	–	–	–	–	–	–	–	–
4.4–4.6	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
4.9–5.1*	+++	++	++	–	–	–	–	–	–	–

* Saline controls.

TABLE 2

Agglutination of peptone broth suspensions of Cl. paraputrificum in serum-acid buffers, the pH of the mixtures being tested potentiometrically

Agglutinations read at three hours

pH RANGE	SERUM DILUTIONS						NO SERUM	pH
	1:20	1:40	1:80	1:160	1:320	1:3200		
6.92–6.96	–	–	–	–	–	–	–	6.63
5.61–5.63	–	–	–	–	–	–	–	5.61
5.09–5.10	–	–	–	–	–	–	–	5.00
4.97–5.00	+++	++	–	–	–	–	–	4.89
4.87	++++	++	++	++	–	–	–	4.80
4.73–4.80	++++	++++	++++	+++	+++	+++	+++	4.67
4.49–4.52	++++	++++	++++	++++	++++	++++	++++	4.42
4.20–4.40	++++	++++	++++	++++	++++	++++	++++	4.35
4.85–5.09*	+++	+++	++	–	–	–	–	4.83

*Saline control.

neutral peptone infusion broth. Normal rabbit serum was diluted in buffer solutions of pH 7.05, 6.06, 5.20, 5.04, 4.85, 4.53, 4.2, and 4.03, respectively. Instead of the complete series of nine dilutions of serum only the first five, namely, 1:20, 1:40, 1:80, 1:160, and 1:320 were tested. A sixth dilution of 1:3200 was included to test for the effect of dilute serum concentration. Controls included buffers without serum and a series of normal serum diluted in physiological saline with a glucose broth culture for the agglutinating suspension. The volumes for these tests were 5.0 cc. serum dilutions and 5.0 cc. of the bacterial suspensions. After mixing and incubating for a half hour, the potentials were measured and the corresponding pH values determined. Part of the mixture in each tube was used for the pH determination and the remainder for agglutination titer. The results are listed in table 2.

Table 2 shows that in a mixture of normal serum and *Cl. paraputrificum* in peptone infusion broth adjusted to different degrees of acidity by buffer solutions the critical pH value for flocculation was between pH 5.0 to 5.10. Above pH 5.10 flocculation did not take place in any tube of the series. In a narrow zone below this value, pH 4.80 to 5.10, only tubes containing the highest concentrations of serum were affected, but below pH 4.80 all tubes showed more or less equal flocculation. The control of serum in physiological saline solution to which glucose broth culture was added showed partial agglutination in serum dilutions up to 1:80.

The complete or nearly complete agglutination of broth suspensions of *Cl. paraputrificum* below pH 4.8 compared with a preliminary observation that watery or saline suspensions of *Cl. paraputrificum* were stable at pH 4.0 indicated that acid alone was not responsible for the non-specific reactions encountered. Therefore, the following experiments were designed to point out other possible factors.

II. OTHER FACTORS RESPONSIBLE FOR NON-SPECIFIC AGGLUTINATION OF BROTH CULTURES OF CL. PARAPUTRIFICUM

Normal serum was diluted as before in known acid buffer solutions and equivalent amounts of the following added: peptone

TABLE 3

Agglutination of Cl. paraputrificum in various media by known acid concentrations

	SERUM DILUTIONS									NO SERUM	pH RANGE
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120		
Peptone meat infusion	–	–	–	–	–	–	–	–	–	–	5.2
	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	4.4–4.6
Meat infusion	–	–	–	–	–	–	–	–	–	–	5.2
	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	4.4–4.6
Peptone water	–	–	–	–	–	–	–	–	–	–	4.7–4.9
	–	–	–	–	–	–	–	–	–	–	4.4–4.6
Uninoculated meat infusion	+++	++	–	–	–	–	–	–	–	–	4.9–5.0
	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	4.4–4.6
Saline	–	–	–	–	–	–	–	–	–	–	5.2
	–	–	–	–	–	–	–	–	–	–	4.4–4.6
Water	–	–	–	–	–	–	–	–	–	–	5.2
	–	–	–	–	–	–	–	–	–	–	4.4–4.6

TABLE 4

Acid agglutinations of sporulating anaerobes

	ACID CONCENTRATIONS EXPRESSED IN pH								
	5.70	5.10	4.80	4.33	3.84	3.45	3.01	2.60	2.20
Cl. Welchii	–	–	++	–	–	–	–	–	–
Cl. Novyi	–	++	+++	++++	++++	++	–	–	–
Cl. septicum	–	–	–	–	–	++	++++	++++	++++
Cl. tetani	–	–	++	+++	–	–	–	–	–
Cl. sporogenes	+++	++++	+++	++++	++++	++++	++++	++++	++++
Cl. botulinum B	–	–	–	–	–	–	–	++++	++++
Cl. bifermentans	–	–	–	–	–	–	–	–	–
Cl. difficilis	–	–	–	–	–	–	–	++++	++++
Cl. paraputrificum	–	–	–	–	–	–	++	++	++
Cl. capitovalis	–	–	+++	+++	++++	++++	++++	+++	++

infusion broth, meat infusion broth, peptone water, saline, and distilled water suspensions of *Cl. paraputrificum*. A control series with uninoculated meat infusion broth was also tested. The final hydrogen-ion concentrations of the mixtures were tested colorimetrically. The significant results obtained with each suspension are listed in table 3.

Table 3 shows that the meat infusion and peptone meat infusion broth suspensions of *Cl. paraputrificum* flocculated in the pH range 4.4 to 4.6, but not at 5.2. The uninoculated meat infusion broth also flocculated in the same range and partially in the serum dilutions 1:20 and 1:40 at 4.9 to 5.0. Suspensions of *Cl. paraputrificum* in water, saline solution, and peptone solution were stable in all tubes. These results suggested that the acid precipitated some substance of the meat infusion, which in turn brought down the bacteria to give the non-specific reactions. Contrary to the observation of de Kruif (1921) the presence of peptone apparently did not extend the zone of acid agglutination. The stability of water and saline suspensions of *Cl. paraputrificum* in the presence of the serum-buffer mixtures indicated that the bacteria were not directly agglutinated by the acid, but it remained to test the influence of acid on *Cl. paraputrificum* without the presence of broth or serum.

III. ACID AGGLUTINATION OF CL. PARAPUTRIFICUM AND OTHER SPORULATING ANAEROBES

Acid agglutination was first utilized by Michaelis (1911) as a possible means of identifying bacteria by their flocculation at the isoelectric points of their specific proteins. He was thus able to distinguish *Eberthella typhosa*, *Salmonella paratyphi* and *Salmonella Schottmuelleri*. His pupil Beniasch (1912) recorded the isoelectric point of a large number of aerobic species of bacteria. Gieszczykiewicz (1916) established acid agglutination values for several of the sporulating anaerobes as follows: *Clostridium tetani*, pH 4.16; *Clostridium botulinum* and *Clostridium chauvei*, pH 3.55; and *Clostridium butyricum*, pH 2.96. The succeeding controversy over the value of this test as a diagnostic procedure was ably reviewed by Fischer (1928), who investigated the acid agglutina-

tion of the hemophilic organisms. He arrived at the same conclusion as many others; namely, the test might apply to some species, but any positive value was limited by the overlapping zones of agglutination of many species.

For the acid agglutinations in the following tests *Cl. paraputrificum* was grown in glucose broth for forty-eight hours, after which it was centrifugalized, then twice washed and centrifugalized, and re-suspended in distilled water. The suspensions were filtered through cotton before using. To 3.0 cc. volumes of this suspension placed in each of nine tubes 1.0 cc. of each known buffer solution was added respectively. The tubes were incubated overnight at 37°C. The same technique was used for the other sporulating anaerobes tested, which were *Clostridium Welchii*, *Clostridium novyi*, *Cl. septicum*, *Cl. tetani*, *Clostridium sporogenes*, *Clostridium bifermentans*, *Cl. difficilis*, and *Cl. capitovalis*. The results obtained are given in table 4.

Table 4 shows that with *Cl. Welchii* only was agglutination limited to a single tube, namely pH 4.80. The others had zones of agglutination ranging from two tubes with *Cl. tetani* and *Cl. difficilis* to nearly complete agglutination in all nine tubes with *Cl. sporogenes*. *Cl. bifermentans* was not agglutinated in any hydrogen-ion concentration studied. The zone of partial agglutination of *Cl. paraputrificum* extended from pH 3.01 to 2.20.

DISCUSSION

Broth cultures of sporulating anaerobes for agglutination tests were used by Robertson (1916), Fildes (1925), and others. Fildes, however, worked with *Cl. tetani* which is non-saccharolytic and cannot give acid agglutination. Hall and Stark (1923) found glucose broth cultures of *Cl. sporogenes* satisfactory for agglutination purposes; later, Hall and Scott (1931) used similar antigens in the serologic study of *Clostridium Sordellii*. Although these two species ferment glucose vigorously, the failure of these workers to encounter non-specific agglutination suggests that the hydrogen-ion concentration of their glucose broth cultures was less than that needed for the acid agglutination of broth cultures of these species.

The fundamental differences between the acid agglutination of Michaelis and the agglutination in the acid-broth-serum-bacteria complex in this study were soon noted. Michaelis used bacteria suspended in distilled water and added acid buffer solutions ranging from pH 3.5 to 5.0. In the present experiments, however, the set-up was that of an ordinary agglutination test; that is, the serum was diluted in either physiological saline solution or buffer solutions from 1:10 to 1:2560 to which were added equivalent amounts of glucose or broth suspensions, doubling the serum dilutions. A control tube contained no serum. Hence, serum and broth with its various components, normal and those of bacterial metabolism, were present in contrast to their absence in the so-called acid agglutination test.

Although the experiments are confined to anaerobic species they illustrate by the overlapping of acid agglutination zones the limitations of Michaelis' test in separating bacterial species. It may be noted also that the agglutination zone, pH 2.60 to 2.20 for *Cl. botulinum* does not coincide with the optimum, pH 3.55, given for this species by Gieszczykiewicz. The optimum for *Cl. tetani* in these results may be at some point between pH 4.33 to 3.84, which would approximate the value, pH 4.16, given by Gieszczykiewicz for this species. The acid agglutination of *Cl. paraputrificum* was far below the acid values tested in the broth series, which further suggests the action is due to the flocculation of some other substance than the bacterial cells to give the non-specific results encountered with glucose broth cultures of *Cl. paraputrificum*.

The influence of serum on acid agglutination was discussed by Michaelis (1917) who claimed that *Escherichia coli*, which was not susceptible to acid agglutination, was flocculated in the presence of normal serum, while *Shigella dysenteriae* was not. Krumwiede and Pratt (1912) showed that typhoid bacteria sensitized with immune serum beyond the titer limits so that no agglutination occurred were agglutinated in lower acid concentrations than unsensitized bacteria. Gieszczykiewicz demonstrated that normal serum in a 1:100 concentration almost eliminated acid agglutination, whereas a 1:1000 concentration exerted no interference.

He also confirmed the observation of Krumwiede and Pratt. In the present experiments it was apparent that serum played little rôle. Only in the narrow zone of pH 4.8 to 5.10 was the combination of broth, bacteria, and normal serum in concentrations greater than 1:80 more sensitive to the action of acid. Outside of these values either complete stability or flocculation was usually noted.

Thus it was apparent that the agglutination of glucose broth cultures of *Cl. paraputrificum* with *Cl. capitovalis* serum which may be considered as equivalent to normal serum, resulted from the action of acid on some substance of the broth infusion, rather than on the serum or bacteria present. Similar reactions were obtained with *Cl. capitovalis* and *Cl. difficilis* which further confirmed the non-specificity of this type of agglutination. These results may explain the discrepancies obtained by Zeissler (1919) and Heller (1920) who condemned the agglutination test for differentiating the sporulating anaerobes.

SUMMARY

Non-specific serum agglutination of glucose broth cultures of *Clostridium paraputrificum* resulted from the action of the acid, produced by the fermentation of the glucose, flocculating in the infusion broth some substance which in turn brought down the bacteria. Acid agglutination of broth cultures of *Cl. paraputrificum* was very close to pH 5.1 in the presence of normal serum in 1:10 dilution, and occurred more or less completely below pH 4.8. The acid agglutination of watery suspensions of *Cl. paraputrificum*, according to the technique of Michaelis, covered a zone of pH 3.01 to 2.20 which further indicated that the non-specific reactions with *Cl. paraputrificum* resulted from the action of the acid on the broth rather than on the bacteria. The non-specific reaction with glucose broth cultures of *Cl. paraputrificum* and other sporulating anaerobes can be avoided by using neutral broth or physiological saline suspensions. It is possible that the non-specific agglutination of acid broth cultures of certain bacteria may explain the failure of some investigators to secure specific reactions in serologic agglutination tests.

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