THE ACID AGGLUTINATION OF PNEUMOCOCCI.*

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The method of agglutination of bacteria by acid was introduced by Michaelis (1) for the differentiation of bacterial species. By means of it closely allied types can be distinguished, since the hydrogen ion concentration at which agglutination is maximal is characteristic for the various species.

The determination of the point of maximal agglutination has been compared to the determination of the melting point of a chemical compound. However, it is not possible to determine accurately the point of maximum agglutination for all cultures. On the one hand, no agglutination at any hydrogen ion concentration may occur. This has been found by Beniasch (2) to be the case for *Bacillus coli,* and, indeed, certain strains of nearly all species of bacteria have been found by Beniasch to be non-agglutinable within the tested reaction limits. On the other hand, agglutination may occur in uniform degree over a somewhat broad range of hydrogen ion concentration.

The result of much work on the acid agglutination of the typhoid-colon group of bacilli has shown that *Bacillus typhosus* and *Bacillus paratyphosus* are easily distinguished by means of the reaction. Differences between the paratyphoid bacilli A and B, or C, have not been seen by most workers (Beniasch (2), Jaffé (3), and Heimann (4)).

The writer wished to learn whether certain types of pneumococci (described by Dochez and Gillespie (5)), which are distinguished sharply by means of serum reactions, but incapable of being distinguished morphologically or by cultural tests, can be distinguished and classified by means of acid agglutination.

THE METHOD OF ACID AGGLUTINATION.

To different samples of bacterial emulsion in distilled water, different hydrogen ion concentrations are imparted, usually varying in

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geometrical progression. The emulsions are incubated at body temperature. Agglutination should occur in some mixtures, and if an optimum is to be recorded, some mixtures should prove to be too acid and others not sufficiently acid for the occurrence of the phenomenon.

The reactions of the bacterial suspensions are controlled by adding to them solutions of a weak acid, or of an acid still further weakened by the presence of one of its salts. By this means low hydrogen ion concentrations are given by relatively high concentrations of acid, so that loss of small quantities of acid in neutralizing alkaline substances from the culture medium is unimportant. In either case the acid concentration is varied in the series of bacterial emulsions; if salt is added the same concentration must obtain in all members of the series. The two equations used for calculating the hydrogen ion concentrations differ only because they are approximations, as the derivation shows.

Applying the mass action law to the electrolytic dissociation of the monobasic acid whose constant is k, we have,

$(H')\cdot(R^-) = k(HR)$ where (HR) indicates the concentration of the acid, in gram molecules per liter, (H′) the concentration of hydrogen ions, and (R^-) the concentration of the R ions. (R) includes all R ions from whatever source. If we increase the concentration of R ions in a solution of the pure acid HR by adding a salt NaR, then we have a molecule of R^- for every molecule of salt introduced, since the salt is almost completely dissociated; and though the dissociation of the acid retrogresses we must have a molecule of R^- for every molecule of H′ split off from the acid. We have, therefore,

$(H')\cdot(\text{salt} + H') = k\,(HR)$ (where salt = the number of molecules of salt introduced per liter)

$= k\,(\text{acid})$ (where acid = the number of molecules of acid introduced),

because the acid is mostly in the undissociated state. Therefore

$$(H') = k\ (\text{acid})/(\text{salt} + H').$$

Now if no salt is present this reduces at once to

$$(H') = \sqrt{k\,(\text{acid})}, \qquad (1)$$

whereas if salt is added even in moderate amounts, (H′)is then small in comparison with (salt), and the equation becomes

$$(H') = k\ (\text{acid})/(\text{salt}). \qquad (2)$$

Equation (2) shows that with this type of regulator dilution does not change, within limits, the hydrogen ion concentration. The k for acetic acid is $1.8 \cdot 10^{-5}$; for lactic acid it is $1.38 \cdot 10^{-4}$. The hydrogen ion concentration is given in grams per liter.

TECHNIQUE.

All the organisms studied had recently been isolated from patients having lobar pneumonia.

The broth used for the tests was a beef infusion containing 0.5 per cent. of sodium chloride and 1 per cent. of Witte's pepton (Kahlbaum), sterilized intermittently in streaming steam for short periods. The reaction was + 0.6 per cent. acid to phenolphthalein.

The lactate mixtures were prepared from stock solutions of one third normal sodium lactate, with a small crystal of thymol, and normal lactic acid, according to the scheme which follows. They were employed fresh.

N/3 sodium lactate in c.c.	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
N lactic acid in c.c.	$\frac{0.12^1}{8}$	$\frac{0.25}{8}$	$\frac{0.5}{8}$	$\frac{1.0}{8}$	$\frac{2.0}{8}$	0.5	1.0	2.0	4.0	8.0	16.0
Distilled water in c.c.	18.4	18.2	18.0	17.5	16.5	18.0	17.5	16.5	14.5	10.5	2.5
Ratio of acid to salt	$\frac{1}{32}$	$\frac{1}{16}$	$\frac{1}{8}$	$\frac{1}{4}$	$\frac{1}{2}$	1	2	4	8	16	32
H ion concentration in grams per liter, multiplied by 10^4	0.04	0.1	0.2	0.4	0.7	1.4	2.8	5.5	11	22	44

A series of dilute lactates (1 to 6) was also used. It was prepared by adding five volumes of distilled water to one volume of each of the lactate mixtures described above.

Normal acetic acid, when diluted (1 to 1.3) by bacterial emulsion in the manner to be described, gives a reaction somewhat less acid than the 32:1 lactate mixture. It was used, and its reaction was recorded as if it were exactly equivalent to that lactate mixture. By diluting normal acetic acid 1 to 2.86 a reaction mixture can be obtained which when diluted by the bacterial emulsion corresponds exactly to the 16 to 1 lactate mixture. By making progressive dilutions from this N/2.86 acetic acid, diluting 1 to 4 in each case, a series of mixtures was obtained corresponding, when bacterial emulsion is added, to

[1] Read 0.12 c.c. of normal acid freshly diluted 1:8.

the lactate mixtures. In all, six different strengths of acetic acid were used.

In two experiments a series of mixtures of sodium acetate and acetic acid was used, each mixture being 1 to 257 normal with sodium acetate. Such solutions are not generally to be recommended. In one other case the salt content was different (table III, footnote 12).

Young cultures were produced by inoculating broth flasks taken cold from the refrigerator and by placing the flasks in the thermostat at 37.5° C. for eighteen hours or less. Cultures thus obtained showed well staining Gram-positive diplococci and short chains of good form. Except in experiment III such young cultures were always used for the tests. All cultures used were carefully examined and found pure. The cultures were whirled at high speed in a centrifuge and the supernatant fluid was poured off, leaving the deposit of cocci with only a small amount of adhering fluid. The cocci were then well emulsified in a volume of distilled water equal to about one tenth of that occupied by the original culture. 0.3 of a cubic centimeter of this emulsion was placed in each of a series of tubes in a rack, and one cubic centimeter of the proper reaction mixture of the regulator to be employed was added to each tube. Tubes containing acetic acid were stoppered. The rack of tubes was then shaken a few times in a standardized manner, because it was found that shaking markedly aids the agglutination, and was then placed in a water bath at about 37.5° C. until agglutination occurred.

EXPERIMENTATION.

The writer found at once that the strains of pneumococcus at his disposal were not readily agglutinated by the acid-salt mixtures (acetic acid, also lactic acid) generally used for the test. Some strains showed no agglutination even after several days at body temperature. Michaelis states that the presence of salts inhibits the acid agglutination of bacteria, but that the effect of salts is slight in concentrations below 1/40 normal. Other investigators apparently have not studied this matter further. The following experiment was performed to see whether in the case of pneumococcus salts can be active in unusually low concentrations.

EXPERIMENT I.

Inhibiting Action of Salts in Low Concentrations.

Pneumococcus 1.

Regulator.	Salt content.	Mixtures incubated.	Ratio acid: salt (lactate).								
			1/8	1/4	1/2	1	2	4	8	16	32
Lactates	N/52	21 hrs.	−	−	−	−	−	−	−	−	−
Lactates diluted 1:6	N/312	3 hrs.	−	−	−	−	+	+±	±	−	−
Equivalent acetic acid	None added	3 hrs.	−	−	−	−	−	++	++	−	−

Pneumococcus 29.

Regulator.	Salt content.	Mixtures incubated.	Ratio acid: salt (lactate).								
			1/8	1/4	1/2	1	2	4	8	16	32
Lactates	N/52	21 hrs.	−	−	−	−	−	−	−	−	−
Lactates diluted 1:6	N/312	21 hrs.	−	−	−	−	−	−	−	−	−
Equivalent acetic acid	None added	3 hrs.	−	−	−	−	−	−	−	+	−
		21[2] hrs.				−	−	−	+	+	−

This experiment shows plainly that the pneumococcus is sensitive to the action of salts in inhibiting acid agglutination even at a normality of 1/312 of salt. This was seen many times in later experimentation.

EXPERIMENT II.

Effect of Washing Pneumococci in Distilled Water.

Pneumococcus 75.

All Mixtures Were Incubated for Twenty-Eight Hours.

	Ratio acid: salt (lactate).								
	1/8	1/4	1/2	1	2	4	8	16	32
Lactate mixture									
Unwashed	−	−	−	−	−	−	−	−	−
Washed	−	−	−	−	−	±	−	−	−
Lactates diluted 1:2.5									
Unwashed	−	−	−	−	−	±	−	−	−
Washed	−	−	−	−	−	+	−	−	−
Lactates diluted 1:6									
Unwashed	−	−	−	−	+	+	+	−	−
Washed	−	−	−	±	±	+±	+±	−	−
Equivalent acetate mixtures									
Unwashed	−	−	−	−	±	+	+	±	−
Washed	−	−	−	−	−	+±	++	±	−

The technique was now controlled to see whether washing the deposit of pneumococci would be a real refinement.

[2] Autolysis took place in the first three tubes during this time.

This experiment shows that no less acid is required to produce a given result[3] when the cocci are washed. Unwashed, though well drained, bacterial deposits were therefore used for all the tests that follow.

Certain results obtained by Dr. Hanes in this laboratory, when compared with the results of the writer, suggested that cultures incubated a few hours longer than was the practice in this investigation agglutinated well in the presence of the usual amount of salt, but at less acid reactions. A flask of broth was therefore inoculated with pneumococcus I and from it in twenty-four hours material was taken and inoculated into a second flask. Both flasks were then incubated for about eighteen hours. Smears showed that the old culture contained many Gram-negative as well as Gram-positive diplococci, whereas the young culture contained only Gram-positive organisms. The acid agglutination test, with undiluted lactates, gave the following results.

EXPERIMENT III.

Age of the Culture.

Culture.	Mixtures incubated.	Ratio acid : salt.					
		1	2	4	8	16	32
Young...	6 hrs.	−	−	−	−	−	−
Young...	20 hrs.	−	−	−	−	−	−
Old	1 hr.	−	−	−	−	−	−
Old	2 hrs.	−	−	±	−	−	−
Old	6 hrs.	−	+	+	−	−	−
Old	20 hrs.	−	+±	++	−	−	−

It will be seen that the old culture agglutinates well in six hours, though the young culture shows nothing in twenty hours, but that the optimum in the case of the old culture is different from that characteristic of the strain and of other organisms of the type (experiment I and table I). The optimum seen here for the old culture coincides with that which Beniasch found for the pneumococcus.

With the technique given above, various strains of pneumococci were now examined by the method of acid agglutination. In accordance with a classification already made for these strains by the

[3] The power of acid regulators to maintain the calculated hydrogen ion concentration in the presence of traces of medium decreases with decreasing concentration of acid in the regulating mixture.

use of immune sera, by protection of mice by the simultaneous administration of serum with living cocci and by agglutination (Dochez and Gillespie (5)), the material is divided into three groups and presented in tables I, II, and III. Many of the strains were at once tested with three regulators (lactates, lactates 1 to 6, and acetic acid). All strains were tested at least once with the undiluted lactates, and when the result was negative a lower salt concentration was tried by the use of the diluted lactates or acetic acid. No strain failed to agglutinate with the acetic acid mixture. Negative results, which were always attributable to too high a salt content of the regulator, have not been recorded in the tables.

TABLE I.

Acid Agglutination of Pneumococci of Type 1 (Neufeld 1).

Culture.[4]	Regulator.[5]	Hours.[6]	Hydrogen ion concentration.[7]								
			0.2	0.4	0.7	1.4	2.8	5.5	11.0	22.0	44.0
1.58^{13}	Lactate 1:6	45	−	−	−	−	−	±	±	−	−
1.58^{16}	Lactate 1:6	2				−	−	+	−	−	−
1.58^{16}	Acetic acid	2				−	−	+	+	−	−
1.64^{7}	Lactate 1:6	3	−	−	−	−	+	+±	±	−	−
1.64^{7}	Acetic acid	3	−	−	−	−	−	++	++	−	−
1.67^{7}	Acetic acid	1 1/2				−	−	+	+	−	−
88.0^{16}	Lactate	6 1/2	−	−	−	−	−	±	±	−	
75.6^{3}	Lactate 1:6	28	−	−	−	−	±	+	±	−	−
75.6^{3}	Acetate	28	−	−	−	−	±	+	+	±	−
94.1^{7}	Lactate	50	−	−	−	−	+	+	−	−	−
94.1^{7}	Lactate 1:6	4	−	−	−	−	−	+±	±	−	−
43.5^{19}	Lactate 1:6	5 1/2				−	−	+	+±	−	−
43.5^{19}	Acetic acid	1 3/10				−	−	+	+±	−	−
73.1^{10}	Lactate 1:6	20				−	−	±	+	−	−
73.1^{10}	Acetic acid	20				−	−	±	++	−	−
73.1^{12}	Acetic acid	1 3/10				−	−	+	+±	−	−
90.1^{10}	Lactate 1:6	1 3/10				−	−	±	±	−	−
90.1^{10}	Acetic acid	1 3/10				−	−	+	+±	−	−
90.1^{11}	Lactate 1:6	1 1/2				±	+	+	+	−	−
90.1^{11}	Acetic acid	1 1/2				−	±	+	+	−	−
44.6^{15}	Lactate 1:6	1 1/5				−	−	+±	+	−	−
44.6^{15}	Acetic acid	1 1/5				−	−	++	++	−	−

[4] The integral part of the laboratory number specifies the strain, the decimal gives the number of animal passages, and the exponent gives the number of transfers since the last animal passage.

[5] These regulators have been described above. Lactate means the sodium lactate-lactic acid mixtures; lactate 1:6 means such mixtures freshly diluted six times with distilled water; acetate means the sodium acetate-acetic acid mixtures.

[6] Time required for the recorded degree of agglutination.

[7] Multiplied by 10^4. The unit is a gram per liter.

In table I are given the results for eight strains of pneumococci of the No. 1 (or Neufeld) type. Only two positive results, and these were weak, were obtained with the undiluted lactate mixtures. This type shows narrow zones of agglutination with an optimum at a hydrogen ion concentration of 5.5 to 11 times 10^{-4} grams per liter. This optimum is therefore a little different from that found by Beniasch (2) for pneumococci.

TABLE II.

Acid Agglutination of Pneumococci of Type 2.

Culture.	Regulator.	Hours.	Hydrogen ion concentration.[8]								
			0.2	0.4	0.7	1.4	2.8	5.5	11.0	22.0	44.0
2.10[5]	Acetic acid	2 3/5				−	−	−	−	+	±
2.10[17]	Acetic acid	1 1/2				−	−	−	+	+	−
95.0[6]	Lactate	17	−	−	−	−	−	−	±	±	±
95.0[7]	Lactate 1:6	50	−	−	−	−	−	±	+	+	+
85.6[9]	Lactate 1:6	4	−	−	−	−	−	−	+	−	−
85.6[11]	Lactate 1:6	20				−	−	±	±	+	±
85.6[11]	Acetic acid	2				−	−	−	+	+	−
85.6[13]	Acetic acid	3				−	−	−	+	−	−
(Same, read later)		22				−	−	−	++	+	±
15.7[12]	Acetic acid	1				−	−	−	+	+	−
23.6[11]	Acetic acid	1				−	−	−	±	±	−
39.5[8]	Acetic acid	5 1/2				−	−	−	+±	+	−
29.7[12]	Acetic acid	3				−	−	−	−	+	−
89.1[9 9]	Lactate	1				+	+	+	+	±	−
89.1[9]	Lactate 1:6	1				+	+	+	+	±	−
89.1[9]	Acetic acid	1				±	+±	+±	+	±	−
89.2[8]	Lactate	1/5			−	±	±	+	+	±	
89.2[9]	Lactate	1/5	−	±	±	±	±	+	+	+	

Table II gives the results for eight strains of pneumococci of a different serological type (No. 2). With the exception of No. 89 these strains showed weak or no agglutination with the undiluted lactate mixture. Some even gave negative results with the diluted lactates. With the same exception the strains show a narrow zone of agglutination with an optimum of 11 to 22 times 10^{-4} grams of hydrion per liter. This lies within the region found by Beniasch to be characteristic for streptococci.

[8] Multiplied by 10^4.

[9] Pneumococcus 89 has constantly shown atypical forms in cultures. Its behavior with serum 2 is, however, perfectly typical.

TABLE III.

Acid Agglutination of Atypical Strains of Pneumococci.

Culture.	Regulator.	Hours.	Hydrogen ion concentration.[10]									
			0.04	0.1	0.2	0.4	0.7	1.4	2.8	5.5	11.0	22.0	44.0
71.1[10]	Lactate	17						−	−	−	+	+	+
71.1[10]	Lactate 1:6	1						−	−	−	+	+	+
71.1[10]	Acetic acid	1						−	−	−	+	+	+
38.6[8]	Lactate	1 1/5						−	−	+	+	+	+
38.6[8]	Lactate 1:6	1 1/5						−	−	+	+	+	+
36.16[3]	Lactate	1						−	−	+	+	+	+
36.16[3]	Lactate 1:6	1						−	±	+	+	+	+
36.16[3]	Acetic acid	1						−	−	+	+	+	+
55.6[11 11]	Lactate	1 1/5						−	±	+	+	+	±
55.6[11]	Lactate 1:6	1 1/5						±	+	+	+	+	+
PaS.1[2]	Lactate	1 1/2						+	+	+±	+±	+±	+
PaS.1[2]	Acetic acid	1 1/2						—	+	+±	++	+	+
62.0[8]	Lactate	2						+	+	+	+	+	+
62.0[8]	Acetic acid	2						±	+	+	+	±	−
37–8.6[5]	Lactate	1 1/5						+	+±	+±	+	±	−
37–8.6[5]	Acetic acid	1 1/5						−	+±	+±	+	±	−
34.6[15]	Lactate	22						+	±	−	−	−	−
34.6[17]	Lactate	45			−	±	+	+	−	−			
76.4[11]	Lactate	2						+	−	−	−	−	−
76.4[11]	Acetic acid	2						+	±	−	−	−	−
76.4[11]	Lactate	1 3/10			+	++	+±	±	−				
82.5[9]	Lactate	22						±	−	−	−	−	−
82.5[11]	Lactate	1 1/2			++	++	±	−	−	−			
52.2[9]	Lactate	6 1/2						+	±	−	−	−	−
52.2[13]	Lactate	1 3/10	+	+	+±	+	±	±	−	−			
Streptococcus mucosus.													
49.3[9]	Lactate	2			+±	±	−	−	−	−	−	−	
19.9[6]	Acetate[12]	1 8/10			−	++	+++	+++	++	−			

Table III contains the results for ten strains belonging to neither type 1 nor 2, and not showing any considerable degree of relationship one to another by serum tests; for one strain (No. 55) which showed relationship with type 2 by the protection test, but not by the agglutination test; and for two strains of *Streptococcus mucosus,* one of which (No. 49) has all the properties of a streptococcus except that it has a large capsule and produces a mucous exudate in mice, the other of which (No. 19) has all the typical properties of a pneumococcus except for its extra large capsule, mucoid growth on

[10] Multiplied by 10^4.

[11] Pneumococcus 55 belongs with type 2 by protection test, but it failed to agglutinate with serum 2.

[12] The concentration of sodium acetate was in this case N/26.

agar, and production of a mucous exudate in mice. The agglutination of most of these organisms is rapid and is never inhibited by salts in the same manner as is that of pneumococci of types 1 and 2. The zones of agglutination are for the most part broad; none are coincident with those characteristic of types 1 and 2.

SUMMARY.

Eight strains of pneumococci of serological type 1, eight strains of type 2, and eleven strains belonging to neither type have been tested by the method of acid agglutination.

Strains belonging to the two typical groups have, as a rule, narrow zones of agglutination. The optimum hydrogen ion concentrations are different in the two cases. Other pneumococci have broad zones or, in a few cases, narrow zones not coincident with those occupied by the typical organisms.

The agglutination of most of the pneumococci of types 1 and 2 is extremely susceptible to the inhibiting action of salts. This is not true of the other pneumococci.

Old broth cultures may show an optimum hydrogen ion concentration different from that shown by young broth cultures.

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