STUDIES IN AGGLUTINATION.

I. The Agglutination of Streptococci.

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INTRODUCTION.

In the immunological reaction of agglutination the character of the environment plays an important part. Among the influential factors are the conditions under which the bacteria are grown, the quality of the bacterial suspensions used, the temperature of incubation of the reaction, the nature and concentration of electrolytes utilized, and agitation or centrifugalization of the serum and bacteria mixtures. It is well known that departures from certain environmental requirements seriously interfere with the validity of the method. Optimal results may be obtained only if the environment be so standardized as to secure the most favorable state for the agglutination of any given organism.

The purpose of the present study is the determination of the more important factors essential for the production of an optimum agglutination balance. The importance and practical value of agglutination in diagnostic and immunological problems give particular impetus to such an investigation.

The mechanism of agglutination is usually conceived of as consisting of two phases: (*a*) union or interaction of antigen and antibody and (*b*) flocculation of the antibody-antigen complex. The first step may be accelerated by appropriate heat incubation (1), by agitation or centrifugalization (1), or by certain critical concentrations of electrolytes (2).[1] Interference with the interaction occurs when

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[1] As for example, the narrow pH zone just above the acid agglutination zone of any given organism, where the agglutinating power of the serum is increased (2).

the heat of incubation is excessive, when the concentration of hydrogen (3) or hydroxyl (4) ion is too high, when polyvalent ions are used as electrolytes,[2] or when such physical obstructive factors as bacterial capsules are present. The second phase is the basis for interpretation; interference with this step will therefore invalidate the method. The bacterial suspension may be too stable or too unstable. In the former case, evidence of antigen-antibody interaction will be lacking (except when cross-adsorption tests are possible), and in the latter case the occurrence of spontaneous or non-specific flocculation makes readings difficult or impossible. Here important factors are the character and concentration of electrolytes employed, and probably, the conditions of bacterial growth.

The optimum agglutination balance is secured, therefore, (1) when conditions are most favorable for antigen-antibody interaction, and (2) when the bacterial suspension is stable enough to prevent non-specific effects, and yet near enough to the flocculation threshold to be brought down by minimum concentrations of specific antisera.

As an approach to the problem, many forms of specific and non-specific agglutination have been studied. Here have been included the effect of hydrogen and hydroxyl ions, of monovalent and polyvalent ions (2, 3, 5–8) and the behavior of autoagglutinating bacteria. In addition, a survey of the methods commonly employed in the agglutination reaction has been made. Colon bacilli and streptococci have been employed chiefly in the experiments, but many other organisms have been used.

The Agglutination of Streptococci.

Most workers with streptococci who have utilized the methods of agglutination and adsorption in their problems have felt that their results might, perhaps, be considered equivocal at times, because of the difficulty of getting satisfactory suspensions of all strains of the organism. The strains vary from extremely granular types, with which it is impossible to work, to entirely satisfactory diffuse ones; and frequently, diffuse strains become granular, and granular ones become diffuse. When one is dealing with granular autoagglutinating strains, it is often impossible to make satisfactory readings of specific agglutination, or to interpret accurately related adsorption reactions. In drawing general conclusions regarding streptococcal types, as in the work on influenzal, empyema, or scarlatinal strains (with which agglutination and adsorption tests are used as the basis of interpretation), it becomes necessary, therefore, that methods be devised for obtaining uniformly stable suspensions of the bacteria.

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[2] Polyvalent ions precipitate the serum (*cf.* $CeCl_3$, personal observation).

In the present paper, a study has been made of the nature of streptococcal autoagglutination. On the basis of the experimental results, general conclusions regarding this phenomenon have been reached, and suggestions for the management of specific reactions have been put forward. The explanation ventured seems to be applicable, perhaps, to similar behavior of other bacteria.

Methods.

Strains of Streptococci.—Hemolytic strains, 10 from throats of scarlet fever, 5 empyema, 1 wound; non-hemolytic strains, 4 septicemia, 1 urine.

Serum.—Rabbit antistreptococcic serum.

Agglutination Methods.—The macroscopic method was used entirely.

Washing of bacteria: Bacteria were washed with distilled water two to four times.

Suspensions: The washed bacteria were suspended in distilled water, and a uniform concentration was sought by the Gates method (9) (opacity to equal a colon bacillus standard of 2 billion per cc.). At times the bacterial growth was small and lighter suspensions were perforce used.

Method of mixing: Equal quantities (usually 0.5 cc.) of the bacterial suspensions to be tested for agglutination, and of the solutions used, were mixed rapidly and thoroughly agitated.

Temperature of reactions: All agglutination tests were incubated in a water bath at 37°C., unless otherwise indicated.

Media.—Stock buffered beef broth, adjusted to a pH of 7.6, or to other pH as indicated.

Temperature of Growth.—Bacteria were grown at 37°C., and at room temperature (17–23°C.). Less bacteria were obtained at the latter temperature, but enough were grown when 40 to 100 cc. of broth were used.

Age of Cultures.—Bacteria were grown 16 to 18 hours; occasionally 24 hour growths were used.

Buffers.—The glycocoll, sodium phosphate, sodium acetate (G.P.A.) buffer recommended by Northrop and De Kruif (6), was found to be satisfactory and was used throughout.

Measurements of Potential.—Potential was determined from the rate of migration, by means of the Northrop cell (5). Calculations were made in accordance with Northrop's formula.[3]

Measurements of Cohesive Force.—Determinations were made by the method of Northrop and De Kruif (6). The du Noüy surface tension apparatus (10) was used.[4]

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[3] Use of the cell was kindly permitted us by Dr. Northrop of The Rockefeller Institute for Medical Research.

[4] Use of the apparatus was kindly permitted us by Dr. Northrop.

RESULTS.

In studying the acid agglutination zone of streptococci, it was noted that the zone varies from day to day, that it varies when the bacteria are grown in broth media of different pH, or in different batches of media, and that the zone varies at times for no accountable reason. It was also noted that there are marked differences in the width of the acid zones of different strains.

Stevens,[5] working in this laboratory, noted last year that granular strains of streptococci (37°C. growth) frequently became diffuse or less granular when incubated at room temperature. A similar related observation has been made for granular diphtheroids by Mellon (11) who believed that he was dealing with pleomorphism, because of the fact that he had two strains of his diphtheroid, one, smooth, predominating at room temperature (20°C.) and the other, granular, at 37°C. Judging from the results of agglutination tests, this does not seem true of the bacteria under our observation.

On the basis of these observations it seemed fair to assume that: (1) cultural conditions, notably thermal or H ion, may be so controlled as to reduce or prevent the tendency to granular growth, and (2) there may be a pH zone in which even very granular strains would remain sufficiently stable for carrying out serum reactions.

Tables I and II show the effect on macroscopic cultural characteristics, on the microscopic appearance of the bacteria, and on the acid agglutination zone of Strain ScB, when the bacteria are grown in broth of varying pH at room temperature and 37°C. respectively. There appear, also, in the tables, the changes in the pH of the broth that occur during growth.

It will be noted from Table I that (1) the growth at 37°C. is granular at lower pH the chains are long and the clumps of bacteria are large and frequent, and the pH of the broth drops a little more than in those grown at room temperature; and (2) growth at room temperature is diffuse in all cases, and the chains are short and the clumps small and infrequent.

From Table II it will be seen that the acid agglutination zone of Group 2 (grown at 37°C.) is regularly wider than that of Group 1, pH 3 to 9 as opposed to pH 3 to 5–6. Study of the rate of aggluti-

[5] Stevens, F. A., personal communication.

nation shows that this is more rapid in Group 2 than in Group 1, and that in Group 2 the rate is faster for the bacteria grown at a lower pH. It will be noted also that agglutination is not complete in Group 2 at pH 6.6 to 9.0. The bacteria grown at room temperature are stable in salt solution, NaCl M/14 (0.425 per cent), while all bacteria in Group 2 agglutinate.

Determinations of the pH at the end of the experiment show no differences sufficient to account for the results (Table III).

TABLE I.

Effect of Growth at Room Temperature and at 37°C., and of Varying pH on the Character of Streptococcus Cultures (Strain ScB).

Condition of growth.	Original pH of broth.	Final pH.	Gross growth characteristics.	Microscopic characteristics.
Bacteria grown 16 hrs. at room temperature.	7.20	6.80	Diffuse.	Chains short; clumps small and rare.
	7.35	7.00	"	" " " " " "
	7.55	7.10	"	" " " " " "
	7.60	7.35	"	" " " " " "
	7.70	7.40	"	" " " " " "
Bacteria grown 16 hrs. at 37°C.	7.20	6.70	Moderately granular.	" long; very many large clumps.
	7.35	6.80	Moderately granular.	" " " " " "
	7.55	7.00	Diffuse.	" " many large clumps.
	7.60	7.00	"	" " " medium sized clumps.
	7.70	7.10	"	" " " " " "

As noted above, the growth at room temperature is usually smaller in amount than that obtained at 37°C. To rule out the factor of earlier stage of growth, in the behavior of the two types, bacteria were grown at 37°C. until their density was the same as that of a room temperature growth begun earlier (density determination by Gates' (9) opacity method). These bacteria remain unstable. Also, in order to get more bacteria, growth was conducted at varying combinations of the two temperatures, starting at room temperature and ending at 37°C., and *vice versa*. The results here are again the same; *i.e.*, once the bacteria have been incubated at 37°C., they become unstable.

TABLE II.

Effect of Growth at Room Temperature and at 37°C., and of Broth of Varying pH on Agglutination of Streptococci at Various pH (G.P.A. Buffer).

Condition of growth.	pH of buffer.	Agglutination.														
		1 hr.					2 hrs.					24 hrs.				
		pH of broth.														
		7.2	7.35	7.55	7.6	7.7	7.2	7.35	7.55	7.6	7.7	7.2	7.35	7.55	7.6	7.7
Group 1; grown at room temperature.	3.0	1−	1	1−	1−	±	1	1+	1	1−	1+	C.	C.	C.−	C.	C.
	4.0	1−	1−	1−	1−	±	1	1	1	1−	1+	C.	C.	C.	C.	C.
	5.0	±	−	±	±	±	±	±	±	±	±	C.	C.−	C.	C.−	3
	6.0	−	−	−	−	−	−	−	−	−	−	C.−	±	C.−	±	±
	6.6	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	7.0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	8.0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	9.0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Distilled H_2O	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	NaCl M/14	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Group 2; grown at 37°C.	3.0	3	3	1	1+	1	C.−	C.−	2	3	2+	C.	C.	C.	C.	C.−
	4.0	3+	3	2	3	2	C.−	C.−	3+	C.−	3+	C.	C.	C.	C.	C.
	5.0	3+	3	2−	3−	2−	C.−	C.−	3+	C.	C.−	C.	C.	C.	C.−	C.−
	6.0	3+	3	±	±	±	C.−	C.−	2	1+	1	C.	C.−	C.−	C.−	C.−
	6.6	3+	3	±	−	−	C.−	3+	2	±	−	C.	3+	3	2+	2
	7.0	3+	2+	±	−	−	C.−	3	2	±	−	C.	3+	3	2+	2
	8.0	3	2+	±	−	−	C.−	3	1+	±	−	C.	C.	C.	3+	3+
	9.0	2+	2	±	−	−	3	2+	1+	±	−	C.	C.	C.	3	3+
	Distilled H_2O	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	NaCl M/14	3	2+	1+	1+	−	C.−	3+	3+	2	1−	C.	C.	C.	C.−	3+

− indicates no agglutination; ±, doubtful agglutination, slightly granular with hand lens; 1, slight definite granular appearance of suspension to the naked eye; 2, marked granular appearance, without flocculation; 3, marked granular appearance, beginning flocculation; C., complete agglutination, supernatant fluid clear. + and − are used to indicate intermediates between these.

Similar acid agglutination zone determinations were now made with several strains, twenty in all. In all cases differences between bacteria grown at room temperature and at 37°C., similar to those noted above, are present. Examples of these differences are presented in Tables IV to VI.

Table IV shows the differences in the acid agglutination zones of strains in which there is a moderate tendency to granular growth, when the bacteria are incubated at 37°C. Table V shows the differences in a very granular strain; it will be seen here that the chief difference lies in the greater rapidity of agglutination of the bacteria grown at 37°C. Table VI shows the results when strains that are regularly diffuse are employed.

TABLE III.

Final pH of Mixtures at the End of the Preceding Experiment.

Readings made at the end of 24 hours.

pH of broth.	Temperature of growth.	Final pH.									
		pH of buffer used in mixtures.								Controls.	
		3.0	4.0	5.0	6.0	6.6	7.0	8.0	9.0	Distilled H_2O	NaCl
7.2	Room temperature.	3.0	4.0	5.0	6.0	6.7	7.0	7.8	8.4	6.6	6.5
	37°C.	3.0	4.0	5.0	5.9	6.6	6.9	7.9	8.4	6.5	6.5
7.35	Room temperature.	3.0	4.0	5.0	5.9	6.6	6.9	8.0	8.6	6.6	6.5
	37°C.	3.1	4.0	5.1	6.0	6.6	7.0	7.9	8.6	6.4	6.4
7.55	Room temperature.	3.1	4.2	5.0	6.0	6.6	7.0	7.8	8.4	6.4	6.4
	37°C.	3.0	4.2	5.0	6.0	6.6	7.0	7.8	8.4	6.4	6.4
7.6	Room temperature.	3.0	4.0	5.0	6.0	6.6	7.1	7.9	8.5	6.4	6.5
	37°C.	3.1	4.0	5.2	5.9	6.6	7.0	7.8	8.4	6.3	6.3
7.7	Room temperature.	3.0	4.0	5.0	6.0	6.6	7.0	7.8	8.4	6.5	6.5
	37°C.	3.0	4.0	5.1	6.0	6.6	7.1	7.9	8.5	6.5	6.4

Tables VII and VIII show the results when *Staphylococcus aureus* and *Bacillus typhosus* are treated in the same way. It will be noted that the stability of the staphylococcus is so increased by growth at room temperature that an acid agglutination zone is practically absent. Only slight differences appear in the acid zones of *Bacillus typhosus*.

TABLE IV.

Determination of the Acid Agglutination Zones of Moderately Granular Strains Grown at Room Temperature and at 37°C.

Readings made at the end of 24 hours.

Temperature of growth.	Strain.	Agglutination.									
		pH of mixtures.								Controls.	
		3.0	4.0	5.0	6.0	6.6	7.0	8.0	9.0	Distilled H_2O	NaCl
Room temperature.	N Y V	C.	C.−	3+	−	−	−	−	−	−	−
	D	C.−	C.	C.	1+	−	−	−	3*	−	−
	1372	C.	C.	C.	1+	−	−	−	−	−	−
	BL	C.	C.	±	−	−	−	−	†	−	−
37°C.	N Y V	C.	C.	C.−	3+	3+	3+	C.	C.	−	C.
	D	C.	C.	C.	C.	C.	C.	C.	C.	−	C.
	1372	C.	C.	C.	C.	C.	C.	2	C.−	−	C.
	BL	C.	C.	C.	C.	C.	C.	C.	†	−	C.

* Agglutination, usually not complete, has been noted many times in this alkaline zone (about pH 8.0 to 10.0). Experiments with G.P.A. buffer have shown that this zone of "alkaline agglutination" is present with many bacteria, *B. typhosus*, colon bacillus, staphylococcus, streptococcus, etc.

† Not done at this pH.

TABLE V.

Determination of the Acid Agglutination Zone of a Very Granular Strain (Strain H) Grown at Room Temperature and at 37°C.

pH of mixture.	Agglutination.							
	Bacteria grown at room temperature.				Bacteria grown at 37°C.			
	1 hr.	2 hrs.	6 hrs.	24 hrs.	1 hr.	2 hrs.	6 hrs.	24 hrs.
3.0	1	3	3+	C.	3	3+	C.−	C.
4.0	1	3	3+	C.	3	3+	C.−	C.
5.0	1	3+	C.−	C.	3	3+	C.−	C.
6.0	1−	2+	2+	C.	3	3+	C.−	C.
7.0	1−	3	C.−	C.	1	3+	3+	C.
8.0	−	2+	C.−	C.	−	2+	3	C.
9.0	−	−	3	C.	−	−	−	C.−
Distilled H_2O	−	−	−	−	−	−	−	−
NaCl	1+	3	C.−	C.	2+	C.−	C.	C.

TABLE VI.

Determination of the Acid Agglutination Zone of a Diffuse Strain (Strain SFI) Grown at Room Temperature and at 37°C.

pH of mixture.	Agglutination.							
	Bacteria grown at room temperature.				Bacteria grown at 37°C.			
	1 hr.	2 hrs.	3 hrs.	24 hrs.	1 hr.	2 hrs.	3 hrs.	24 hrs.
2.8	±	1−	1	C.−	1	2	2+	C.
3.6	±	1−	1	C.−	1	2−	2	C.
4.0	−	±	1−	C.−	−	1−	1	C.
4.4	−	±	1−	1	−	±	1−	C.
4.8	−	−	−	−	−	−	−	1
6.0	−	−	−	−	−	−	−	−
7.0	−	−	−	−	−	−	−	−
Distilled H_2O	−	−	−	−	−	−	−	−
NaCl M/14	−	−	−	−	−	−	−	−

TABLE VII.

Determination of the Acid Agglutination Zone of Staphylococcus aureus Grown at Room Temperature and at 37°C.

Readings made at the end of 24 hours.

Temperature of growth.	Agglutination.								
	pH of mixtures.								
	2.8	3.2	3.6	4.0	4.4	4.8	5.2	5.6	6.0
Room temperature.	1	−	−	−	−	−	−	−	−
37°C.	C.	C.	C.	3	C.	±	−	−	−

TABLE VIII.

Determination of the Acid Agglutination Zone of B. typhosus Grown at Room Temperature and at 37°C.

Readings made at the end of 24 hours.

Temperature of growth.	Agglutination.								
	pH of mixtures.							Controls.	
	2.8	3.2	3.6	4.0	4.4	4.8	5.2	Distilled H_2O	NaCl
Room temperature.	C.	C.	±	−	−	−	−	−	−
37°C.	C.	C.	C.	±	−	−	−	−	−

One experiment with *B. typhosus* showed no difference in the agglutination zones.

Table IX shows the effect of room temperature and 37°C. growth upon spontaneous agglutination when NaCl is the electrolyte employed. Further experiments with NaCl and K_2SO_4 show similar results, although in these no agglutination occurred in the room temperature growth except in the tubes containing saturated salt.

Table IX shows the effect of NaCl. It will be noted that there is no agglutination in the room temperature growth except at a concen-

TABLE IX.

Agglutination of Streptococcus (Strain 1372) in Various Concentrations of NaCl, When Grown at Room Temperature and at 37°C.

Readings made at the end of 24 hours.

Temperature of growth.	Agglutination.						
	Concentration of NaCl.						
	M/2	M/4	M/8	M/14	M/56	M/224	0
Room temperature.	—	—	—	—	3	—	—
37°C.	±	3+	C.—	C.	C.—	—	—

TABLE X.

Agglutination of Streptococcus (Strain ScB) in Various Concentrations of K_2SO_4, When Grown at Room Temperature and at 37°C.

Readings made at the end of 24 hours; reaction at room temperature.

Temperature of growth.	Agglutination.							
	Concentration of K_2SO_4.							
	Saturated.	M/4	M/12	M/36	M/108	M/327	M/981	0
Room temperature.	C.	—	—	—	—	—	—	—
37°C.	C.	C.	C.	C.	C.	C.	C.	—

tration of M/56, while the bacteria grown at 37°C. agglutinate in M/4 to M/56 concentrations. Table X shows the results when K_2SO_4 is employed as electrolyte.

Summing up the results of these experiments, we note that (1) streptococci grown at room temperature (17–23°C.) (*a*) are stable in all concentrations of NaCl ordinarily employed in the agglutination reaction and (*b*) possess an acid agglutination zone resembling

that of stable organisms in general; and (2) streptococci grown at 37°C. (*a*) are not stable in NaCl solutions, and (*b*) have either a widened acid agglutination zone or (*c*) if this zone is not widened, are unstable in the buffer solutions outside this pH zone for other reasons. This latter alternative would seem to be the correct one. Results presented further on would seem to show that the reason is a difference in the attracting forces.

In their recent work on the stability of bacterial suspensions (2, 3, 5–8), Northrop and De Kruif consider agglutination in terms of two forces: a repelling force which keeps the bacteria apart,—charge on the bacteria,—and an attracting or cohesive force. They have shown that the potential (charge on the bacteria) is the main factor in suspensions when the salt concentration is low (less than 0.01 N) and that the cohesive force plays the important part in higher salt concentrations. They have shown that, provided the cohesive force is unaffected, bacteria agglutinate when the potential lies between −15 and +15 millivolts; that is to say, when the cohesive force is large enough to overcome the repelling one. And they have shown that high concentrations of salts (over 0.01 N) reduce the cohesive force, and with this the critical potential agglutination zone.

Accordingly, in seeking for an explanation of the results above, it becomes necessary to look for differences of potential or cohesion, or both, in the two groups.

In considering the question of potential, differences must be investigated in suspensions containing salt in low concentration, to eliminate the cohesive factor. When high valency ions are employed, the effect on the potential can be obtained in high dilution.

Tables XI and XII show the effect upon agglutination and potential (Strain ScB) when thorium nitrate is used. The bacteria were first set up in varying dilutions to determine the point at which agglutination occurs (Table XI). As will be noted, this takes place from 5×10^{-5} to 6×10^{-6}.

Then, dilutions from 3×10^{-4} to 1.1×10^{-6} were taken and agglutination and potential determined. Table XII gives the results.

It will be seen that in very low concentration (Tubes 1 to 3) the bacteria retain their usual negative charge, and that in higher concentration (Tubes 4 and 5) the charge becomes positive. Complete agglutination occurs in Tube 3 only, P.D. −11 and − 14 respectively; in Tubes 2 and 4 very doubtful flocculation occurs. This indicates

that the critical potential (agglutination) zone of the bacteria grown at room temperature lies somewhere between −17 and +17, and that of the 37°C. growth between −19 and +24. The difference between the two critical potential zones is thus very small, indicating that it is a very slight factor, if any. Determinations of potential of bacteria suspended in buffer solutions show similar minor differences.

TABLE XI.

Agglutination of Streptococci (Strain ScB) in Various Concentrations of Thorium Nitrate, When Grown at Room Temperature and at 37°C.

Readings made at the end of 24 hours; reaction at room temperature.

Temperature of growth.	Agglutination.					
	Molar concentration of $Th(NO_3)_4$.					
	5×10^{-4}	1.6×10^{-4}	5×10^{-5}	1.8×10^{-5}	6×10^{-6}	2×10^{-6}
Room temperature.	−	−	C.	C.	−	−
37°C.	−	−	C.	C.	C.	−

TABLE XII.

Agglutination and Potential of Streptococci (Strain ScB) in Various Concentrations of Thorium Nitrate, When Grown at Room Temperature and at 37°C.

Experiment at room temperature; agglutination readings made at the end of 24 hours.

Tube No.	Dilution of $Th(NO_3)_4$.	Bacteria grown at room temperature.		Bacteria grown at 37°C.	
		Agglutination.	Potential.	Agglutination.	Potential.
1	1.1×10^{-6}	−	−24	−	−23
2	2.2×10^{-6}	±	−17	±	−19
3	4.5×10^{-6}	C.	−11	C.	−14
4	9.0×10^{-6}	±	+17	±	+24
5	1.8×10^{-5}	−	+26	−	+29

Early in the investigation it was felt that the cohesive factor is the probable explanation of the differences between the room temperature and 37°C. growths.

In favor of this conjecture may be noted the following facts.

1. If complete agglutination be taken as the criterion for the acid agglutination zone (Tables II, IV, V, and VI), the zones of the two groups are practically the same. This observation is against a potential and in favor of a salt effect.

2. If we assume, from the results of the NaCl and K_2SO_4 experiments (Tables IX and X), that the cohesive force of the bacteria grown at the higher temperature is greater, the whole mechanism of the difference is explained. Reference to these tables shows that there is agglutination in high salt concentration in the 37°C. growth, and none in the other. Salt in these concentrations reduces the charge on the bacteria to limits well within the critical potential (agglutination) zone, but usually markedly depresses the cohesive force, and accordingly agglutination fails to occur (6). This, as noted, is true of the bacteria grown at room temperature; that is to say, cohesive force is reduced by the high salt concentration. If, however, the cohesive force is high or resistant to the salt effect in this zone of low potential, the bacteria will agglutinate; or, to put it another way, agglutination in this low potential zone, when high salt concentration is used, can only occur if the cohesive force remains high. This, as noted, occurs with the bacteria grown at 37°C.

3. The acid agglutination zone of staphylococci (Table VII) is virtually abolished by room temperature growth; that is to say, growth at this lower temperature either reduces cohesion or makes this force less resistant to salt effects; and additional lowering by the fairly high salt concentration of the buffers prevents agglutination in the acid zone in which the low potential usually leads to agglutination, and in which it leads to agglutination with the bacteria grown at 37°C.

On the basis of this reasoning, differences in the cohesive force of the two groups of bacteria would seem to be the probable explanation of the findings. Direct measurements of this force were now made and the results obtained confirm the supposition. As noted by Northrop and De Kruif (6), their method of determination of cohesive force is reasonably accurate; and in our hands, after the technique had been learned, the method, though not one of absolute precision, gave surprisingly parallel as well as reproducible results.

A brief review of the method devised by Northrop and De Kruif (6) may be given here.

Thick films of the bacteria are made on a heavy glass slide and on a cover-slip. These are allowed to dry and are then passed rapidly through a flame a few times to ensure adhesion to the glass. The slides are then placed in the dish of the du Noüy surface tension apparatus, film to film, in the solutions whose effect is to be tested, and after they have been left in contact 1 minute (no pressure is used) the force required to pull the films apart is measured.

Table XIII shows the effect of concentrated NaCl upon the cohesive force of the bacteria grown at room temperature and at 37°C. Measurements were made of the cohesive force, in distilled water, and in

concentrated NaCl (1.7 M), for Strain ScB. As will be noted, the cohesive force of the bacteria grown at room temperature is reduced by the concentrated salt; while that of the 37°C. growth is not only not reduced, but is actually slightly increased. Experiments with other films and with films of another strain (No. 1372) gave parallel, though not always such striking results.

From the results above, it seems reasonable to conclude that many strains of streptococci, when grown at the temperature of incubation employed as routine (37°C.), possess an attracting or cohesive force which is resistant to the depressing effects of the concentrations of salts commonly used as electrolytes, and on this account spontaneous

TABLE XIII.

Determination of the Cohesive Force of Smears of Bacteria Grown at Room Temperature and at 37°C.

Temperature of growth.	Solution in which smears were placed.	Readings on dial (torsion of wire).	Average.	Force required to separate 2 sq. cm. surface.
				mg.
Room temperature.	Distilled H_2O	15, 20, 19, 18	18	90
	NaCl 1.7 M	12, 13, 9, 10	11*	55
37°C.	Distilled H_2O	22, 20, 21	21	105
	NaCl 1.7 M	19, 30, 31, 30	27.5	137.5

* After immersion in NaCl solution, this was washed out with distilled water, and after resuspension in distilled water the cohesive force between the smears was reread and averaged 17.5 (87.5 mg.).

agglutination occurs.[6] Moreover, growth at a lower temperature in some way prevents this resistance, with resulting greater stability of suspensions.

When one approaches the problem of securing suspensions of streptococci that are satisfactory for use in agglutination reactions, the following facts must be noted.

[6] A number of workers with streptococci have overcome the difficulty with autoagglutinating strains by using low concentrations of NaCl as electrolytes. The probable explanation of their success lies in the fact that, with low salt concentration, the potential is high (5), and that then the repelling effect involved overcomes the high cohesive force and the bacteria become stable.

1. Many strains are habitually diffuse and always give satisfactory stable suspensions.

2. Many strains are habitually or intermittently granular, but by growth at room temperature may be made diffuse, and are then satisfactory to work with. Table XIV shows a specific agglutination test with a strain (No. 1372) belonging to this group. The electrolyte used was NaCl M/14 (0.425 per cent). It will be noted that the room temperature growth is stable in the high dilutions of serum and in the control, and that the agglutination titer is clear-cut; and that the 37°C. growth is unstable, complete agglutination occurring in all tubes, including the control.

TABLE XIV.

Specific Agglutination of Streptococci (Strain 1372) Grown at Room Temperature and at 37°C.

Electrolyte, NaCl M/14.

Temperature of growth.	Agglutination.									
	Serum dilution.								Controls.	
	0.1	0.01	0.005	0.0025	0.0012	0.0006	0.0003	0.00015	0	Distilled H_2O
Room temperature.	C.	C.	C.	C.	C.	C.–	2	–	–	–
37°C.	C.	C.	C.	C.	C.	C.	C.	C.	C.–	–

3. A few strains are always granular, even when grown at room temperature. A number of methods have been devised for coping with this difficulty. Northrop and De Kruif (12) have suspended washed bacteria in 0.001 N NaOH, and have found such a suspension stable when NaCl in low concentration (M/320) is used as electrolyte. Several observers, working with autoagglutinating bacteria, both streptococci and other organisms, have obtained stable suspensions by using very low concentrations of electrolytes; the probable explanation of their success has been noted above.

Table XV shows the application of these methods to a fairly granular strain. It will be noted here that the bacteria become stable in NaCl concentrations over 0.01 N, and that the bacteria treated with NaOH agglutinate more slowly than the untreated ones. It is probable

that the required concentration of electrolyte for stability will vary with different strains, becoming less as the strain is more granular.

Observations on the acid agglutination zones of streptococci (Tables II, IV, and V) show that the bacteria are more stable at pH 7 to 8. For this reason a very granular strain (Strain ScBF, scarlet fever) was tested against a specific scarlet fever streptococcus serum (No. 1372) in G.P.A. buffer, pH 7.0. Table XVI shows the results. It will be seen that agglutination is very slow in the control tube and quite marked in the tubes containing serum. Specific agglutination is therefore easily to be recognized on the basis of

TABLE XV.

Stability of Granular Streptococci (Strain ScB) in Various Concentrations of NaCl.

Condition of growth.	Time.	Agglutination.						
		Concentration of NaCl.						
		3 M	1 M	0.33 M	0.11 M	0.037 M	0.012 M	0.004 M
	hrs.							
Bacteria suspended in distilled H_2O and added to NaCl solution.	1	−	−	−	−	−	−	−
	2	±	±	1−	1	±	−	−
	4	1	2	2+	3	+	−	−
	24	C.−	C.	C.	C.	±	−	−
Bacteria suspended in 0.001 N NaOH and added to NaCl solution.	1	−	−	−	−	−	−	−
	2	−	−	−	−	−	−	−
	4	−	±	±	−	−	−	−
	24	+	1	±	±	±	−	−

early readings, but would be impossible from a 24 hour reading. Similar results were obtained at pH 8.0. When NaCl (M/14) is used as electrolyte the agglutination in the control is too rapid for satisfactory comparative readings.

In view of the evidence that salts in low concentration favor stability, and in view of the observation (Table XIII) that there is an increase of the cohesive force of bacteria grown at 37°C. in the presence of salts in high concentration, washed bacteria were next tested for stability in G.P.A. buffer, pH 7.0, in lower concentration. The results appear in Table XVII, and show that greatest stability is obtained in low concentrations (less than M/100).

Stability was also sought for, but without success, by the use of NaCl, $BaCl_2$, and $CaCl_2$ in high concentration (saturated to M/10).

Finally (Table XVIII), the agglutination titer of specific serum, with the various methods above, was investigated. Granular growths, incubated at 37°C., of a streptococcus (Strain ScB) were

TABLE XVI.

Specific Agglutination of a Granular Streptococcus (Strain ScBF from Scarlet Fever) with Heterologous Serum (No. 1372) in Buffer Solution (G.P.A.) pH 7.0.

Bacteria grown at room temperature.

Time.	Agglutination.						
	Serum dilution.					Controls.	
	0.05	0.005	0.0025	0.0012	0.0006	No serum.	Distilled H_2O
hrs.							
1	1	1	1	1	1+	—	—
2	2+	3+	2+	2+	2+	±	—
3	3	C.—	3+	3+	3+	1	—
24	C.	C.	C.	C.	C.	3+	—

TABLE XVII.

Stability of Streptococci (Strain ScB) in Various Dilutions of G.P.A. Buffer, pH 7.0.

Time.	Agglutination.				
	Total salt concentration.				
	M/25	M/50	M/100	M/200	M/400
hrs.					
1	1	±	—	—	—
2	2	1	—	—	—
4	3	1+	±	—	—
24	C.	C.—	1	—	—

used, with a stable growth, made so by incubation at room temperature, of the same organism as the control. It will be noted that the titer of the serum is not appreciably affected (Table XVIII).

Taken together these experiments indicate that some strains of streptococci are always diffuse and give stable suspensions for specific agglutination; some are granular, but by growth at room temper-

ature may be made diffuse and will then yield satisfactory suspensions; and the few remaining refractory granular strains may be made less granular by growth at room temperature, and can then be tested satisfactorily with electrolytes in low concentration (especially when treated with NaOH), or else in buffer solutions of pH 7.0 to 8.0.

TABLE XVIII.

Specific Agglutination Titer of Antistreptococcus Serum with a Granular Strain (Strain ScB), with Various Electrolytes.

Readings made at the end of 24 hours.

Bacteria.	Electrolyte.	Agglutination.							
		Serum dilution.							Control.
		0.01	0.005	0.0025	0.0012	0.0006	0.0003	0.0001	
Granular streptococcus grown at 37°C.	NaCl M/14	C.	C.	C.	C.	C.	C.	C.	C.
	NaCl M/56	C.	C.	C.	C.	2	1	1	1
	NaCl M/56 + NaOH 0.001 N	C.	C.	C.	C.	2	±	±	±
	G.P.A. buffer. pH 7.0, M/25	3	C.	C.	C.−	2	1	1	1
	M/50	C.−	C.	C.	C.	2	2	1	1
	M/100	C.−	C.	C.	C.	2	1	±	±
Control, diffuse. Room temperature growth.	NaCl M/14	C.−	C.	C.	C.−	2	±	−	−
	Stock broth.	C.	C.	C.	C.	C.−	2	2	2

SUMMARY.

1. The spontaneous agglutination of streptococci has been studied.

2. This spontaneous agglutination would seem to be caused by the presence of a bacterial cohesive force higher than that usually found when bacteria are suspended in salt solutions of the concentration commonly employed as electrolyte in specific agglutination reactions.

3. Many granular autoagglutinating strains of streptococcus may be made diffuse by growth at room temperature (17–23°C.) and then lose their tendency to agglutinate spontaneously.

4. All factors that reduce cohesive force or that make the repelling force relatively greater than the cohesive force make for stable suspensions.

5. Methods for management of the specific agglutination of refractory autoagglutinating strains of streptococci have been presented.

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