A CLASSIFICATION OF NON-HEMOLYTIC STREPTOCOCCI.

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No group of bacteria has been suspected of etiologic relationship with so many and diversified disease processes as the streptococcus. At the same time there is no group which suffers more confusion in the relationship of its numerous members. The purpose of the present work was to attempt a classification of twenty-eight strains of non-hemolytic streptococci by a comparative study of their biochemical and immunological reactions.

Since the streptococcus was first recognized as an infectious agent by Fehleisen (1) in 1882, various methods of classifying these organisms have been proposed. The object of every attempt to classify has been to determine whether all streptococci are identical with accidental differences in pathogenicity and cultural features or whether the differences noted constitute more or less fixed characteristics, which demand separate grouping for such members. All the schemes can be included in those dealing with morphology, the ability to produce special lesions, and immunological and biochemical reactions.

Classification on the basis of morphology was abandoned early because of the influence which cultural environment was found to have on morphology. The terms *longus* (2), *brevis*, and *conglomeratus* (3) are now considered merely descriptive of growth phenomena and not of fundamental properties.

The opinion that certain streptococci have a definite etiologic relationship to certain diseases was natural, since they were frequently associated with these diseases. Thus Fehleisen (1) assumed a certain variety to be the cause of erysipelas, while Rosenbach (4) was the first to call attention to the importance of the variety which he called *pyogenes*, in the production of local purulent processes. Similarly, scarlet fever and smallpox were regarded as being of streptococcus origin. This supposition, however, was weakened by the failure to establish

experimental proof of their etiologic rôle in these diseases, and further, by the finding of streptococci in the throat, gastro-intestinal tract, and feces of normal individuals. It might be noted that while formerly a special streptococcus was thought to be etiologically responsible in a given disease, many modern believers in the unity of streptococci prefer to regard them all as identical and their capacity of producing any given disease as merely an accidental and temporary property.

The relation of streptococcus to disease as well as the relationship of different streptococci to one another was at first based upon the results of agglutination reactions. Van de Welde (5), the first to study this reaction, reported (6) a specific agglutination between a univalent serum and its homologous streptococcus. Baginsky and Sommerfeld (7) found in the serum of scarlet fever patients specific agglutinins for streptococci isolated from the throats of such patients. Similar findings were reported by Hasenknopf and Salge (8) and Moser and von Pirquet (9). De Waele and Sugg (10) isolated streptococci from the blood of cases of smallpox both before and after death and found that the sera of smallpox patients agglutinated the variety of streptococcus obtained, while sera from unvaccinated individuals failed to give positive reactions. Soon, however, discordant reports appeared. Meyer (11) tested ten streptococci against four monovalent sera obtained by immunizing rabbits with four of the strains, and found that cross agglutination occurred. Nevertheless, the organisms from purulent processes were not affected by sera immune to streptococci from the throats of scarlet fever and rheumatism patients. From these observations he concluded that agglutination reactions could be used to separate the streptococci of angina from those of septic processes. Other workers then began to report promiscuous cross agglutination between the various streptococci. Weaver (12), investigating the serum of scarlet fever patients, concluded that the agglutination reaction in this disease was in no way specific. Neufeld (13) thought that, in certain cases at least, agglutinability varied inversely with virulence. Since most of the strains that he used were capable of being made highly virulent for white mice it now seems probable that they were of the hemolytic variety. Aronson (14), investigating the value of serum in the treatment of so called streptococcus infections, concluded that a polyvalent serum was necessary on account of the failure of one streptococcus from a given disease to be agglutinated by a serum produced by immunizing with another streptococcus from the same disease. He thus argued against the constancy of streptococci as specific producers of disease. Marmorek (15) reviewed the investigations that had been made up to 1902 and concluded that all human pathogenic streptococci were the same. This view was later supported by Zoeppritz (16). Fischer (17) tested twenty-one streptococci from various pathological and normal sources against sera immune to eight representative members of the series and obtained such promiscuous cross agglutination that he decided a great multiplicity of strains existed, and that the agglutination reaction was not effective in grouping them.

From the foregoing it will be seen that the question of classification was unsettled and no plan for grouping streptococci had found wide acceptance. Two courses of procedure were open. One, the result of the failure of previous work, followed the idea that all streptococci were the same, and that the differences which the various workers had endeavored to interpret as fixed characteristics were in reality the fleeting impress of chance environment. At the same time it was asserted that these inconstant organisms produced a great variety of diseases, and to explain this varied activity the theory of elective localization was formulated. This postulates transmutations to a surprising degree of frequency, and often the agency which endows a streptococcus with affinity for the stomach, or for the heart valves, or for the joints, operates in the same focus of infection, the tonsil. Proof has been offered in the production in animals of corresponding lesions in which no factor other than the activity of streptococci is considered. Inasmuch as it is insisted that the properties of elective localization are apt to be lost in the process of isolating a streptococcus, and that the first culture even though mixed must be used, this theory in its present form eludes accurate experimental demonstration.

The other course was to attack the problem from the standpoint of biochemical reactions. Gordon (18) studied the fermentation reactions of streptococci on certain test substances. This line of work was soon taken up by others, and Andrewes and Horder (19) reported extensive studies on 1,200 strains and succeeded in placing the so called pathogenic streptococci in four groups. They found that outside of the first group called *pyogenes*, which was composed largely of streptococci from purulent processes, the source of the streptococci did not accurately determine their place in the groups. In analyzing the results of the reactions, the different groups are found to contain from 14 to 42 per cent of variants. Objections to these biochemical tests on the grounds of inconstancy were advanced by Ritchie (20), Walker (21), and others. However, the discrepancies were elicited by doing the tests under diverse conditions of age, viability, and culture media, and do not discredit the method as much as they indicate the necessity for uniformity in performing the tests. The reasons for variation have been discussed by Thro (22, 23) and Broadhurst (24). Very recently Holman (25) suggested a more extensive differentiation of the entire streptococcus group, based upon their hemolytic and methemoglobin production properties and upon their fermentation of lactose, mannite, and salicin. Sixteen groups are described, but as so few carbohydrates are employed no variants of these groups are recognizable.

Andrewes and Horder found agglutination reactions "troublesome and disappointing," but attempts were soon directed to a correlation of fermentation and agglutination reactions. Floyd and Wolbach (26) concluded that fermentation reactions could be used to separate streptococci into large groups, while agglutination reactions merely emphasized the individuality of members. Kligler (27), on the other hand, found that agglutination reactions ran strikingly parallel with fermentation reactions.

Although Schottmüller (28) in 1903 emphasized the difference between streptococci on the basis of their action upon blood agar plates, very little attention is paid to this factor in the above reports, except for Holman's work. Most workers agree in establishing a strong connection between a certain number of purulent infections and the hemolytic streptococcus (*Streptococcus pyogenes*). It is not unreasonable to assume that much of the cross agglutination reported was due to the fact that one type of streptococcus, the hemolytic, was tested, because the source of many of the strains was a purulent process. Analysis of the reports of Meyer (11) and Neufeld (13) illustrate this point. Analyzing the report of Kligler, in which attention was paid to the hemolytic property of the strains, we find support for the argument that the property of producing hemolysis is important. Kligler tested a serum produced by immunizing a rabbit against a hemolytic strain and found that it agglutinated ten out of twenty-nine hemolytic strains, none out of seventeen "green" strains, and five out of twelve indifferent strains. Thus this serum displayed a marked capacity to agglutinate hemolytic strains; a marked capacity to agglutinate indifferent strains; but no capacity to agglutinate green strains. Two sera immune to green strains agglutinated two out of twenty-nine hemolytic strains and seven out of seventeen green strains. Thus their agglutinating capacity was marked for green but weak for hemolytic strains.

These brief considerations make it logical to require that the hemolytic and non-hemolytic groups should be studied separately in an attempt to classify, and for this reason in the present work the study was limited to non-hemolytic strains alone.

EXPERIMENTAL.

In studying the organisms on which this report is based, attention was paid to the source, growth in plain broth, effect on ascitic-dextrose-agar, solubility in ox bile, action on red cells, according to the method used by Lyall (29), and virulence. In addition, the fermentation reactions were compared with the agglutination and complement fixation reactions.

Methods.

1. For testing the effect of streptococci on red cells, dilutions of a 24 hour broth culture were made in a row of small tubes, using plain broth as a diluent. Each tube contained 0.5 cc. of culture dilution, the doses being graduated as follows: the first tube, 0.5 cc. of culture; the second, 0.25 cc.; the third, 0.12 cc., etc. To each tube 0.5 cc. of 5 per cent saline suspension of sheep red blood corpuscles was added. The mixtures were read after incubation in the water bath, at 37°C. for 1 hour. Streptococci either (1) hemolyze the cells, (2) produce methemoglobin in the unhemolyzed cells, or (3) have no effect upon the cells. The method is not accurately quantitative, but the results are more nearly comparable than are those obtained with the blood agar plate method. However, for comparison both methods were employed.

2. For testing virulence, white mice were inoculated with 18 hour broth cultures in amounts of 2.0 cc., 1.0 cc., and 0.1 cc. If an organism was lethal at 0.1 cc. it was titrated further.

3. Fermentation reactions were done with litmus milk and with raffinose, inulin, salicin, and mannite as test substances. Media containing these were prepared by adding 1 per cent of the test substance to Hiss serum water. In examining the effect of the streptococci on these carbohydrates, a tube of each was inoculated with about 0.2 cc. of actively growing broth culture, incubated, and observed for 10 days.

4. For the animal immunization, a rabbit was used for each of the twenty-eight streptococci. Immunization was effected by injecting the animals intravenously with saline suspensions of killed streptococci at 4 day intervals, in doses equivalent to 10 cc. of broth culture. The injections were continued until the serum of an animal gave marked complement fixation with an antigen made from the corresponding streptococcus. Most of the animals produced good complement-fixing antibodies after six to ten injections.

The sera from the same animals were used for both complement fixation and agglutination reactions, and sera of the same or nearly the same dates were used for both reactions. In the case of complement fixations, as soon as a serum showed definite fixation with its

own antigen, it was tested on the same day with the same materials, hemolytic system, etc., against the twenty-eight antigens, and those antigens which gave fixation were noted. A few days later the animal was bled again and the serum was titrated against those antigens with which fixation was previously obtained. The above precautions were taken because it was noticed, in a few instances, that an old serum tended to give fixation with an increased number of antigens. In performing the agglutination reactions, a dextrose broth culture of a streptococcus was tested against the twenty-eight sera. The reason for this arrangement is obvious, since it was desirable to perform both tests with sera obtained at the same bleeding. All of the twenty-eight rabbits could not be immunized at the same time, and while the age of the serum does not affect agglutinating capacity, complement fixation reactions must be performed with fresh serum because variations in tests showed this to be advisable. Accordingly, while all the sera were on hand when the agglutination tests were made, owing to the uncertainty of keeping rabbits alive over a long period, complement fixation tests had to be made as soon as an animal's serum showed sufficient immunity.

5. In the complement fixation reactions various constituents were used in the following quantities: 0.1 cc. of streptococcus antigen, two units of complement and anti-sheep amboceptor, and descending amounts of immune serum, as 0.1 cc., 0.05 cc., 0.025 cc., etc. The complement-antigen-serum mixtures were made up to 1.5 cc. and incubated in the water bath, at 37°C. for 1 hour. Sensitized cells prepared by mixing 0.5 cc. of amboceptor dilution and 0.5 cc. of a 5 per cent suspension of cells were added, and the tubes again placed in the water bath at 37°C. for 1 hour before reading.

The antigens were prepared as follows. Washed sediment of a 24 hour broth culture was suspended in 5 cc. of saline, and 5 cc. of absolute alcohol were added. The resulting precipitate was separated by centrifugalization and desiccated *in vacuo*. It was then ground and weighed. 10 mg. were dissolved in 5 cc. of a 2 per cent antiformin solution in the water bath at 56°C. and the solution was neutralized with 0.1 N sulfuric acid, with litmus paper as an indicator. The free chlorine was liberated by adding one or two drops of 5 per

cent sodium thiosulfate, the end-result being tested with potassium iodide starch paper. The solution was made up to 10 cc. with carbolized saline, and centrifugalized. 1 cc. of the antigen then represented 1 mg. of dried ground precipitate. This method was always followed. It therefore seems reasonably certain that the antigens in these experiments represent a constant amount of their corresponding streptococci.

6. Agglutination reactions were performed by making dilutions of serum in 0.5 cc. of dextrose broth, so that the first tube contained 0.1 cc. of serum; the second, 0.05 cc.; the third, 0.025 cc., etc. To each tube 0.5 cc. of a 24 hour dextrose broth culture was added. The mixtures were placed in the water bath at 37°C. for 2 hours, and readings were made after they had stood in the ice box over night.

RESULTS.

Table I contains a list of the organisms, their source, and the main characteristics as outlined above. Each of the twenty-eight strains was obtained from one colony.

It will be seen that with the exception of one organism (B1), which was possibly a contamination, all may be said to have come from pathological sources. Two diseases, acute rheumatic fever and bacterial endocarditis, are chiefly represented. In the case of tonsillitis, the streptococci indicated were from the predominating colonies. All are true streptococci as can be seen from their morphology, their action on ascitic-dextrose-agar, and their insolubility in bile. Those which produce methemoglobin in the presence of red blood cells are called green. Three of the streptococci were indifferent in their action upon red cells. The group from bacterial endocarditis is striking for its lack of virulence, while the rheumatism group is consistently more virulent. Attempts to raise the virulence of a few of the streptococci by passage through white mice were unsuccessful.

Table II shows the results of the fermentation reactions. All the organisms were tested soon after isolation. Thus the original tests were not all made at the same time or with the same lot of media. The final tests were performed at the same time and with the same

TABLE I.

Streptococcus.	Source.	Appearance in broth.	Length of chains. Diplococci.	Effect on ascitic-dextrose-agar.	Solubility in bile.	Effect on red blood cells.	Lethal dose for white mice.
1 A65	Blood culture; acute arthritic rheumatism.	Turbid (diplococci).	2–6	Precipitate.	Insoluble.	Green.	0.1 cc. of 24 hr. broth culture.
2 59F	"	"	6–20	"	"	"	0.1 cc.
3 A49	"	"	6–20	"	"	"	0.1 "
4 B38	"	"	4–20	"	"	"	Not fatal at 1 cc.
5 B39	"	Semiturbid; granular sediment.	4–20	"	"	"	" " " 2 "
6 A135	"	Turbid.	2–8	"	"	"	" " " 2 "
7 38D	"	"	8–10	"	"	"	0.1 cc. of 24 hr. broth culture.
8 A141	Blood culture; rheumatic endocarditis.	"	6–20	"	"	"	2 cc.
9 A119	"	"	2–8	"	"	"	Not virulent at 2 cc.
10 A179	Culture from heart valve; P. M. rheumatic endocarditis.*	"	6–12	"	"	"	2 cc.
11 B26	Acute tonsillitis.	Coarse diplococcus; turbid.	10–30	"	"	"	Not tested.
12 B23	"	Turbid.	10–20	"	"	"	Not fatal at 2 cc.
13 K	"	"	10–20	"	"	"	0.1 cc.
14 A102	Sputum; lobar pneumonia.	Coarse diplococcus; turbid.	4–20	"	"	"	0.5 "

15	B1	Surface colony on plate; acute arthritic rheumatism.	Coarse diplococcus; turbid.	10–30	Precipitate.	Insoluble.	Green.	1 cc.
16	A140	Knee joint; P. M. chronic arthritis.	Turbid.	4–10	"	"	"	0.1 cc.
17	A84	Blood culture; P. M. bacterial endocarditis.	Coarse diplococcus; turbid.	4–20	"	"	"	0.5 "
18	A4	Blood culture; A. M. bacterial endocarditis.	Turbid.	6–20	"	"	"	Not fatal at 2 cc.
19	B4	"	"	2–8	"	"	"	"
20	A148	"	"	2–8	"	"	"	"
21	XK	"	"	2–8	"	"	"	"
22	A30	"	"	10–50	"	"	"	"
23	R	"	"	4–10	"	"	"	"
24	B29	"	"	2–6	"	"	"	"
25	O	"	Clear medium, soft sediment.	10–30	"	"	"	"
26	A26	"	Diplococci, bacillary shapes; semiturbid; clumps.		"	"	Indifferent.	"
27	A56	"	Clear, clumps; granular sediment.	2–6	"	"	"	"
28	MB	"	Clear; granular sediment.	2–6	"	"	"	"

*P. M. indicates postmortem; A. M., antemortem.

TABLE II.

Streptococcus.		Interval between 1st and 2nd tests.	No. of generations between 1st and 2nd tests.	Fermentation reactions.				
				Milk.	Raffinose.	Inulin.	Salicin.	Mannite.
		mos						
1	A4	19	10	+*	—	—	—	—
2	O	15	4	+	—	—	—	—
3	XK	8	5	+	—	—	—	—
4	59F	30	15	+	—	—	—	—
5	A49	18	4	+	—	—	—	—
6	A141	15	5	+	—	—	—	—
7	A179	14	5	+	—	—	—	—
8	B39		One test.	+	—	—	—	—
9	A30	18	10	+	+	—	—	—
10	R	13	7	+	+	—	—	—
11	A65	16	5	+	+	—	—	—
12	B29	5	2	+	+	—	—	—
13	A148	14	5	+ +	— +	+ —	+ —	— —
14	A102	15	4	+	+	—	+	—
15	B38		One test.	+	+	+	—	—
16	B23	5	3	+ +	— +	+ +	+ +	+ —
17	K†	15	6	+ +	+ —	— —	+ +	— —
18	MB	8	4	+	—	—	+	—
19	38D			+	—	—	+	—
20	B26	5	8	+	—	—	+	—
21	A140	15	5	+ +	— —	+ —	+ +	— —
22	A119	16	6	+	—	+	+	—
23	B4	8	2	+	—	+	+	—
24	B1	8	7	—	—	+	+	—
25	A26	18	9	+	—	—	+	+
26	A135	16	6	+	—	—	+	+
27	A56	19	8	+	+	+	—	+
28	A84			+ +	+ —	— —	+ +	— +

*+ indicates acid and clot; —, no acid or clot.

† The second test of No. 17 K is probably incorrect as it was recently found that the original Strain K was lost.

media. This may be an important factor in interpreting variations. The arrangement followed in the table is based on the final tests. If an organism showed a variation on second test, the test was done

three times. In five instances, the first and second tests varied. Accordingly in these five the first reading represents one test, and the second reading represents three tests.

It will be seen that all the strains can be placed in two groups of the Andrewes and Horder classification; namely, the *fecalis* group, the five mannite fermenters, and the *salivarius* group which embraces the remaining twenty-three. The results agree with those of the above mentioned investigators in showing that the source is not a determining factor in the grouping. These streptococci could be subdivided into more groups than merely those of *salivarius* and *fecalis*, if the classification advised by Holman could be applied. But this requires testing the fermentation capacities of the various strains with lactose. This was not done. No general conclusions can be drawn from this table. It merely shows that most of the organisms belong to a group called *salivarius*, the feature of which is the fermentation of raffinose but which is permitted to contain variants that do not ferment this substance. With a series of only twenty-eight members a grouping on the basis of fermentation is not conclusive since we have to place members of widely varying fermentative activity together, in a grouping which investigation on a larger scale shows to be logical.

The results of the complement fixation reactions are striking. In the early part of this experiment, the sera tested showed a wide variation in fixing capacity. Thus, while one serum would fix only its own antigen, another would fix many more, and the results seemed accidental and confused. As the work proceeded, however, it became evident, first, that, in general, where a serum gave extensive cross fixation, its corresponding antigen was fixed by very few sera, and then only by sera of equally wide fixing capacity; and second, though to a less noticeable degree, that the sera which gave fixation with a small number of antigens corresponded to antigens which gave fixation with a large number of sera. In other words, there was an inverse ratio between the fixing capacity on the part of a serum and the capacity to be fixed on the part of the corresponding antigen. Inspection of Table III will make this clear. Serum 3 has wide fixing capacity; Antigen 3 is fixed only by sera which are nearly identical in fixing capacity with Serum 3. Again, Serum 28 gives exten-

sive fixation but Antigen 28 is fixed only by Serum 28. On the other hand, Sera 8, 11, 14, and 20 are very limited in fixing capacity; the corresponding antigens, however, are fixed by many sera.

This phenomenon occurred with sufficient regularity to constitute a fact. It is important to determine its cause. The explanation adopted was based on a consideration of the complexity of the different streptococci. It was thought that some streptococci might be more complex than others, perhaps in the structure of their chemical nucleus. If a rabbit is injected with such a streptococcus, its serum will present an antibody capable of fixing this streptococcus, and such other streptococci as contain the same or some of the same structural units. But if a rabbit is injected with an organism containing only one of these units, it will present an antibody incapable of union with the more complex structures and will fix only streptococci of corresponding simplicity. This led to the placing of some antigens above others in the table, and to the formation of subgroups.

Table III illustrates these points. Sera 3 to 7 have large fixing capacity. But Antigens 3 to 7 are fixed only by sera which have nearly equal fixing capacity: *viz.*, Nos. 3 to 7. This for the time being is called Group I. Besides fixing its own antigens this group of sera fixes Antigens 8 to 16. Of these, Nos. 8 to 12 may be said to constitute a separate group, first, because Antigens 8 to 12 are fixed by Sera 3 to 7, and second, because Sera 8 to 12 show similar though not identical relationships with antigens. Antigens 13 to 16 as well as Sera 13 to 16 show new relationships, and constitute an intermediate group, because by antigen and by antibody they are related to Groups I and II. Inspection of the table will show that these connections are irregular, but attention must be paid to these subgroups, because in a larger series of organisms such subgroups may assume large proportions. In a small series like the present one, irregular members in the intermediate positions are to be expected. Antigens 21 to 28 as well as the corresponding sera seem to constitute a group independent of Group I which is called Group II. In this group also tendency to subgroup formation can be readily seen, and no doubt, in a larger series, Nos. 22, 24, and 26 might be the heads of a number of identical organisms. In fact, No. 28 is the

only serum of sufficiently wide reacting capacity to deserve an independent grouping in the way that Sera 3 to 7 are distinct. Nos. 17 to 20 are called Group IIa because they are closely related to Group II, both by antigen and antibody, and are intermediate members because of their relations with Groups Ia and Ib. Finally Group II is seen to be connected with Group I through Nos. 1 and 2. The naming of groups is arbitrary and merely for the purpose of distinction.

The complement fixation reactions on which this grouping is based showed some variation, where the tests were repeated; but the variations that occurred were such as not to affect the grouping of the streptococcus in question. Thus Serum 11 fixed Antigens 11 and 12, while the serum of another rabbit immune to No. 11 fixed Antigens 10, 11, and 12. In like manner, two rabbits were immune to Streptococcus 3 and their sera differed only in the intensity of fixation with Antigens 11, 13, and 21. Such variations may be incidental to uncontrollable factors in the complement fixation reaction.

The sera of twelve of the twenty-eight rabbits, including Nos. 2 and 21, were found to give negative reactions before immunization was begun and hence the non-specific fixation sometimes ascribed to normal rabbit serum cannot be a factor in these tests.

It will be noted that while an antigen is usually fixed more completely by its homologous serum, the rule does not always apply, as for example, Strain 22. The same also applies to the action of sera. Some rabbits yielded sera of weak fixing power, and one, No. 18, yielded serum which gave agglutination but not complement fixation reactions.

In the table agglutination reactions outside the zone of complement fixation are indicated by an A.

Table IV shows the results of the agglutination reactions, which were less satisfactory. A strongly positive reaction was not always present between serum and the corresponding streptococcus, and cross reactions were fewer. Agglutination occurred in some cases where there had been no complement fixation reaction, but, as Table III indicates, this apparent discrepancy does not interfere with the grouping effected by complement fixation reactions.

TABLE III.

Results of Complement Fixation Reactions.

Rabbit serum.	Streptococcus antigens.																												
	1 A4	2 A26	3 K	4 A56	5 MB	6 A84	7 A140	8 38 D	9 A102	10 A119	11 B4	12 A148	13 B1	14 B23	15 A141	16 A135	17 XK	18 A30	19 A179	20 R	21 A65	22 59F	23 A49	24 O	25 B29	26 B38	27 B39	28 B26
1 A4	4 4 4	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
2 A26	4 4 4	4 4 4	o	o	4 3 1	3 3 2	1 1 o	o	o	o	o	o	o	3 2 1	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
3 K	o	3 3 2	4 4 4	4 4 4	3 4 4	3 3 3	4 2 o	4 4 4	4 4 4	4 4 4	4 2 2	4 4 4	3 3 2	4 4 3	4 4 3	o	o	o	o	o	o	o	o	o	o	o	o	o	2 1 o
4 A56	o	2 1 o	2 1 o	4 4 4	4 4 4	3 1 o	4 4 2	4 4 1	4 3 1	4 4 1	4 4 4	4 4 3	3 3 1	4 4 3	4 4 3	4 3 2	o	o	o	o	o	o	o	o	o	o	o	o	o
5 MB	o	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	1 1 o	4 4 4	4 4 4	4 4 4	o	o	o	o	o	o	o	o	o	o	o	o	o
6 A84	o	3 1 o	3 1 o	4 4 1	4 4 1	4 4 4	4 3 1	4 3 1	4 3 1	4 3 1	4 3 1	4 3 1	o	4 3 1	3 2 1	o	o	o	o	o	o	o	o	o	o	o	o	o	o
7 A140	o	o	3 o o	4 4 4	3 o o	o	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	o	3 o o	3 o o	o	o	o	o	o	o	o A	o A	o	o	o	o	o	o
8 38D	o	o	o	o	o	o	o	4 4 4	o	o	o	o	o	o	o A	o	o	o	o	o	o	o	o	o	o	o	o	o	o
9 A102	o	o	o	o	o	o	o	4 4 2	4 4 4	4 4 4	4 4 4	4 4 4	o	o A	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
10 A119	o	o	o	o	o	o	o	4 1 o	4 1 o	4 3 3	4 4 2	4 4 2	o	o A	o A	o	o	o	o	o	o	o	o	o	o	o	o	o	o
11 B4	o	o	o	o	o	o	o	o	o	o A	4 4 4	2 2 2	o	o A	o A	o A	o	o	o	o A	o	o	o	o	o	o	o	o	o
12 A148	o	o	o	o	o	o	o	o	o	2 3 2	4 4 1	4 4 2	3 2 o	o	o A	o	o	o	o	o	o	o	o	o	o	o	o	o	o
13 B1	o	o	o	o	o	o	o	4 4 3	1 4 4	1 3 2	1 1 1	2 3 2	4 4 4	o	o	o	o	o	o	4 4 4	o	o	o	o	o	o	o	o	o

14 B23	o	o	o	o	o	o	o	o	2 3 1	o	o	o	o	4 4 4	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
15 A141	o	o	o	o	o	o	o	4 4 4	o	4 4 4	3 1 o	4 4 4	o	o	4 4 4	o	o	o	o	o	o	4 4 3	4 4 o	o A	o	o	o	o	o	o
16 A135	o	o	o	o	o	o	o	o	o	o	o A	o A	o	o	o	4 4 4	o	o	o	o A	o	o	o A	o	o	o A	o	o	o	o
17 XK	o	o	o	o	o	o	o	o	o	o	3 2 o	4 2 o	o	4 3 1	o	o	4 3 2	o	o	4 3 2	4 2 1	o	o	o	o	o	o	o	o	o
18 A30	o	o	o	o	o	o	o	o	o	o A	o A	o A	o	o	o A	o	o	o A	o	o A	o A	o	o	o	o A	o A	o	o	o	o
19 A179	o	o	o	o	o	o	o	o	o	o	o	o	o	4 4 4	o A	o	o	o	4 4 4	o	o	o A	o A	o	o	o	o A	o	o
20 R	o	o	o	o	o	o	o	o	o	o	o A	o	o A	o	o	o	o	o A	o	4 4 4	o	o	o	o	o	o	o	o	o	o
21 A65	o	o	o	o	o	o	o	o	o	o	o A	o	o	o	o	o	o	o A	o	o A	4 4 1	o	o	o	o	o	o	o	o	o
22 59F	o	o	o	o	o	o	o	o A	o	o	o	o	o	o	o A	o	o	o	o	o	o	4 4 4	4 4 4	o	o	o	o	o	o	o
23 A49	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o A	o	o	o A	o	o	o	4 4 4	4 4 4	o	o	2 2 o	o	o	o	o
24 O	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o A	o	o	o	o	4 4 4	o	o	o	4 4 4	1 2 4	4 4 4	3 4 4	o	o	o
25 B29	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	4 4 4	o	4 4 4	o	o	o	4 4 4	4 4 4	4 4 4	4 4 4	o	o	o
26 B38	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	4 4 4	o	o	4 4 4	o	o	o	o	o	4 4 4	2 2 1	o	o	o
27 B39	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o A	o	4 4 4	o	4 3 2	4 4 4	o	4 2 1	1 1 o	o	o	4 4 2	4 4 1	o	o	o
28 B26	4 4 4	o	o	o	o	o	o	o	o	o	o	o	o	4 4 3	o A	4 4 3	4 4 3	4 4 3	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	4 4 3	4 4 4	4 4 4	4 4 4

4 = complete fixation. 3 = 25 per cent of cells hemolyzed. 2 = 50 per cent of cells hemolyzed. 1 = 75 per cent of cells hemolyzed. o = no fixation. $\overset{o}{A}$ = agglutination but no fixation.

The numbers indicate plus signs, and the reading from above downward indicates the following dilutions: the top figure in each group, a dilution of 0.1 cc.; the second figure, 0.05 cc.; the third, 0.025 cc. Only three readings are inserted on account of lack of space. Five dilutions were used in each test.

TABLE IV.

Results of Agglutination Reactions.

Rabbit serum.		Streptococci.																												
		1 A4	2 A26	3 K	4 A 56	5 MB	6 A 84	7 A140	8 38D	9 A102	10 A119	11 B4	12 A148	13 B1	14 B23	15 A141	16 A135	17 XK	18 A30	19 A179	20 R	21 A 65	22 59F	23 A49	24 O	25 B29	26 B38	27 B39	28 B26
1	A4	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2	A26	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3	K	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
4	A56	−	−	−	+	−	−	−	−	−	−	−	−	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−
5	MB	−	−	−	+	+	−	−	−	−	−	−	−	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−
6	A84	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
7	A140	−	−	−	−	−	−	+	−	−	±	−	−	−	−	−	−	−	−	−	−	−	±	+	−	−	−	−	−	−
8	38D	−	−	−	−	−	−	−	+	−	−	−	−	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−
9	A102	−	−	−	−	−	−	−	−	+	+	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
10	A119	−	−	−	−	−	−	−	−	−	+	+	+	−	+	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−
11	B4	−	−	−	−	−	−	−	−	−	+	+	+	−	+	±	±	−	−	−	+	−	−	−	−	−	−	−	−	−
12	A148	−	−	−	−	−	−	−	−	−	−	+	+	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−
13	B1	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
14	B23	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
15	A141	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	+	−	−	−	−	−	−
16	A135	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	+	−	−	−	+	−	−	+	−	−	+	−	−	−
17	XK	−	−	−	−	−	−	−	−	−	−	±	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
18	A30	−	−	−	−	−	−	−	−	−	+	+	+	−	−	±	−	−	+	−	+	+	−	−	−	+	+	−	−	−
19	A179	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	−	−	−	+	−	−	+	+	−	−	−	+	−	−
20	R	−	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−	+	−	+	±	−	−	−	−	−	−	−	−
21	A65	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−	+	+	−	−	−	−	−	−	−	−
22	59F	−	−	−	−	−	−	−	±	−	−	−	−	−	−	±	−	−	−	−	−	−	+	+	−	−	−	+	−	−
23	A49	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	−	−	+	−	−	−	+	+	−	−	−	−	−	−
24	O	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	−	−	−	−	−	−	−	−	+	−	−	−	−	−
25	B29	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
26	B38	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
27	B39	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	−	−	−	±	−	−	−	+	−	−	−	+	+	
28	B26	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	−	−	−	−	+	−	−	−	−	−	−	−	+	

+ indicates marked agglutination; ±, moderate agglutination; −, no agglutination; o, test not done.

If greater reliance could be placed in the results of the agglutination tests, contrast could be pointed out between the form of the chart of agglutination reactions, and that of complement fixation reactions. This is especially noticeable in the upper left-hand corner where sera with wide fixing capacity have very narrow agglutinating capacity. But it is better to await a similar finding in the study of other groups of bacteria, as well as more definite knowledge concerning the mechanism of the two reactions in question.

It will be noticed that there is no agreement between the grouping based on fermentation reactions and that based on agglutination reactions. For example, Strains 38D and B26 ferment the test substances in the same way; yet they occupy widely separated places in the chart of agglutinations. The same can be said of Strains A26 and A135 as well as of A65 and B29. It is probable that fermentation tests and agglutination tests are instruments which measure two widely different activities of streptococci; so that classifications based on these two tests have little common ground for comparison.

DISCUSSION.

The simplest observation that may be made on examining the chart of complement fixation reactions is that there is a right-sided element and a left-sided element, together with a group in the midzone, where these elements mingle. In any arrangement of the chart, Serum 3 will always occupy a position on the side opposite the side on which Serum 28 will be. While it is true that study of more streptococci might reveal strains different from those herein tabulated, it is not probable that the general form of the chart would be changed, because there are at present no elements suggestive of a new kind of variation which further study would accentuate. A repetition of this entire experiment with the same streptococci would probably not alter the form of the chart in any way because similar reactions were found in those cases where the immunization was repeated with fresh animals and the tests were done a second time.

The next observation to be made, and one which was made first in the present work, is that the strains seem to differ in complexity

with corresponding difference in their antisera. Thus a strain which produces an antiserum of wide reacting capacity, will not itself react with a serum of narrow capacity; and a strain which produces an antiserum of narrow reacting capacity will usually react with many sera of wider reacting capacity than its own. This is the inverse ratio described above.

The work of others with other kinds of bacteria, while not expressing it directly, can be interpreted in such a way as to support both these observations. This should be the subject of another discussion. It will suffice to say that classifications of gonococci and typhoid bacilli can be arranged to show both the tendency to right-sided and left-sided features as well as the inverse ratio referred to above. Previous classifications of streptococci have not embraced more than twelve strains with corresponding antisera.

The explanation of these observations must be theoretical because the mechanism of the complement fixation reaction has not been completely determined.

If, hypothetically, we regard this reaction as an adsorption phenomenon (30), depending on differences in surface charge as well as on chemical surface configuration (31), for the combination of its different factors, it may be possible to refer to the strains on the left side of the chart as being composed of protein molecules of opposite charge to those on the right side. The complex strains would be said to be made up of several kinds of molecules of a certain charge; the simple ones of only one or two molecules of the same charge. The mid-zone would contain those strains which partake of both features. If the antisera be thought of as containing proteins of a charge opposite to that of their corresponding antigens, the failure to react with antigens of different charge can be understood. Finally, the antiserum for a complex antigen would present a complex surface configuration, while that for a simple antigen would show a corresponding simplicity.

In this way the chart of complement fixation reactions might be regarded as outlining the sphere of variation of the non-hemolytic streptococcus with regard to this one reaction.

CONCLUSIONS.

1. No connection can be demonstrated between grouping of non-hemolytic streptococci based upon fermentation reactions, and grouping based upon immunological reactions.

2. A classification of non-hemolytic streptococci can be effected by studying the complement fixation reactions between the streptococci and their antisera.

3. The arrangement of the streptococci in such a classification depends on the fact that two diverse elements are present in the group. Some strains partake entirely of one of these elements, some entirely of the other, while other strains partake of both.

4. The arrangement is further determined by the fact that among the strains composed of one element there are differences in complexity, some strains being made up of many molecules or features, others having much simpler structure. This gives rise to an inverse ratio between the fixing capacity of a serum and the capacity of the corresponding antigen to be fixed.

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