

## STUDIES ON INDIFFERENT STREPTOCOCCI.

### I. SEPARATION OF A SEROLOGICAL GROUP—TYPE I.

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In the search for the etiological agent of rheumatic fever, attention has frequently been called to the possibility that streptococci may in some way be concerned in the production of the disease. Mostly the suggestions have dealt with organisms of the *viridans* group, less frequently with the hemolytic group, but only recently has it been proposed by Small (1) and Birkhaug (2) that the indifferent streptococci must be considered in this connection. This newer view-point is explainable when it is remembered that the bacteria isolated during life or at necropsy from patients with the disease have usually been *Streptococcus viridans*, and that the demonstration of the indifferent varieties has been a much less common occurrence.

The existence of the indifferent streptococci has been a matter of common knowledge for some decades.

Mandelbaum (3), who mentioned their occurrence in the throat and elsewhere, regarded them as simply a variety of saprophytic organism. Zangemeister (4), commenting upon their lack of virulence, considered them apparently as a degraded form of *S. hæmolyticus*, and believed that under proper conditions certain of them could assume the properties of the latter organism. Their cultural characteristics were dealt with by Brown (5), and a tentative classification upon the basis of certain fermentation reactions was advanced.

That the indifferent streptococci were not completely apathogenic was suggested by the observations of Kinsella (6), who recovered them in four instances from the blood stream of patients suffering from subacute bacterial endocarditis. Furthermore, Rosenow (7) on a few occasions isolated them from joint punctates of acute rheumatic fever patients. Working with a strain grown from the blood of a patient suffering from the latter disease, Small (1) demonstrated that inoculation of this organism intravenously into rabbits resulted in the production of lesions which strikingly resembled those described by Bracht and Wächter (8)

and by Thalhimier and Rothschild (9) and Cecil (10), and others following similar injections of *S. viridans*. Treatment of patients with an antiserum prepared with this strain produced effects recalling those reported two decades ago by Menzer (11) and others as resulting from the treatment of acute rheumatic fever patients with polyvalent antistreptococcus serum. More recently Birkhaug (2), Kaiser (12), and later Swift, Wilson, and Todd (13), have called attention to the fact that certain strains of indifferent streptococci possessing in common the property of fermenting inulin yield culture filtrates which on intradermal injection in moderate dilution produce reactions in individuals suffering from rheumatic fever as well as from other conditions. Perhaps the most striking observation in this connection is the fact that heating this filtrate in boiling water for 1 hour augments its "toxicity" (13). Birkhaug has also suggested that his inulin-fermenting organisms show a certain degree of serological interrelationship.

In order for different workers in a field such as this to be able to compare results satisfactorily, some sort of classification of the organisms in question is desirable. Despite the difficulties encountered by others in classifying streptococci of the hemolytic and *viridans* types, it seemed of interest to examine a number of strains of indifferent streptococci, and to determine whether any simple serological grouping was possible. For this purpose 159 strains were secured. Sixteen of these were obtained through the kindness of Dr. Birkhaug, including the R F 1 strain with which much of his work was carried out. These were all inulin-fermenting organisms of the type described in his paper. Later, as the work was in progress, Dr. Small kindly forwarded two cultures, R 1 and R 9; the former was that strain originally isolated and described by him (1). The remaining 141 were secured from the throats of patients suffering from various diseases, including rheumatic fever, from the throats of normal individuals, or from the interior of tonsils removed at operation. Two of our strains were isolated from blood cultures of patients with acute rheumatic fever.

#### *Methods.*

Culture material was obtained from the throats with sterile swabs and from the interior of excised tonsils after sterilization of the surface, and the initial seeding was made upon plain blood agar plates. After an incubation period of 24 to 36 hours colonies of indifferent streptococci were transferred to plates of Birkhaug's medium (2) from which the bile was omitted. These plates served as a rough means for the separation of the inulin-fermenting and non-fermenting types. From these plates strains of both varieties were chosen, and subsequently were

kept in stock culture in blood broth. Final determinations of the capacity of the organisms to ferment inulin and salicin were carried out with tubes of Hiss serum water. Incubation was prolonged for 10 days before negative results were recorded.

Antisera were prepared by the intravenous inoculation into rabbits of whole 24-hour cultures in phosphate broth pH 7.8 containing 0.05 per cent of dextrose. The injections were given semiweekly over a period of 6 to 8 weeks. During the first 2 weeks amounts of 0.5 cc. and 1 cc. respectively of heat-killed culture were administered. Thereafter living culture was employed in doses of 0.5 cc., 1.0 cc., and finally of 2.0 cc. The latter dose was repeated in case trial bleedings failed to reveal a satisfactory titer of agglutinin. The animals were exsanguinated 8 or 9 days following the last dose. By this means it was a simple matter to obtain sera which would agglutinate the homologous organisms in dilutions of 1:5000.

For carrying out agglutinations, the bacteria were grown for 24 hours in phosphate broth pH 7.8 containing 0.05 per cent dextrose. Usually the resulting culture formed a homogeneous, turbid suspension, which for the purpose in hand was extremely satisfactory. In a few instances concentration with the centrifuge was necessary to obtain a sufficiently dense suspension; and occasionally growth was so granular as to render the culture useless for agglutination. Antisera were diluted 1:125, 1:250, 1:500, and 1:1000 in phosphate broth; 0.5 cc. of each of these dilutions was mixed in small tubes with 0.5 cc. of bacterial culture; all tests were recorded in terms of the final dilution of serum which resulted. On each test day a series of similar dilutions of normal rabbit serum was employed with each strain under investigation. Suspension controls were set up, in which 0.5 cc. of culture was mixed with 0.5 cc. of phosphate broth. All tubes were kept in an incubator at 56° for 3 hours, at the end of which time readings were recorded.

Antigens for use in the precipitation test were prepared as follows: 35 cc. of a 24-hour growth in 1 per cent dextrose broth were centrifuged, and the sedimented organisms were suspended in 10 cc. of  $N/100$  NaOH in physiological salt solution. In a few instances in which growth was light, as estimated roughly from the volume of the packed organisms, smaller amounts of the alkaline solution were used. These suspensions were permitted to stand overnight in the ice box, at the end of which time they were centrifuged. The clear supernatant fluid was acidified with 10 per cent acetic acid, whereupon a rich precipitate of acid-insoluble protein appeared. This was permitted to flocculate overnight in the ice box, and was then centrifuged off. The water-clear supernatant fluid was neutralized with  $N/1$  NaOH, and was then ready for use. In carrying out the tests 0.1 cc. of antiserum was placed into each of a series of small tubes, and the antigen was added in amounts of 0.2, 0.1, and 0.025 cc.; sufficient normal salt solution was added to make the final volume 0.5 cc. A control series was always employed in which the same amounts of antigen and salt solution were added to 0.1 cc. of normal rabbit serum. The tubes were kept in the water bath at 37°C. for 2 hours, following which they were placed in the ice box; readings were made the following

morning. The resulting precipitates were found to have gathered in the bottom of the tubes in the shape of coherent discs, which strongly resembled those produced when pneumococcus specific soluble substance is mixed with its corresponding antiserum; this suggested the possibility that substances of similar nature were extracted by this simple procedure from the indifferent streptococci.

Parallel agglutination and precipitation tests were carried out as above outlined with each of the 159 strains. At first there were employed four sera, prepared respectively with two of Dr. Birkhaug's strains, and two of our own. Inasmuch as it was soon found that interchangeable results were obtained with all four sera, it was considered permissible to perform all of the agglutination tests with a single serum (R 867), and all of the precipitations with another (R 862). These sera were of approximately the same titer. The first was prepared against Strain Q 88 E, which was isolated from the blood stream of a patient with rheumatic fever. The second was prepared against Strain RF 24 T, secured from Dr. Birkhaug and originally isolated by him from the throat of a patient with the same disease. Results were recorded in the usual way with + signs, + + + + being the maximum; and except for a few instances which will be considered below the results of the two tests were found to check rather closely.

#### RESULTS.

In Table I are given in summary the results of the observations upon these 159 strains. In the group designated "Inulin fermentation prompt," acid and clot were formed in the Hiss serum water within a period of 48 hours. The group designated "Inulin fermentation delayed" required longer periods of time, and occasionally clot formation remained in abeyance.

As the result of these observations it is possible to separate the strains into two large groups. The first consists of those organisms which show strong agglutination and precipitation reactions with the sera employed. This serological uniformity seems sufficient warrant for the inclusion of all these strains within a single category, which may be designated as Type I. As might be expected, the organisms are not absolutely identical, as revealed by minor variations in the strength of the reactions. Included under Type I are a few strains which fail to conform exactly to the above conditions: (1) some which show spontaneous agglutination, but which can be typed by means of the precipitin reaction, (2) others which agglutinate to titer in the Type I serum, but from which it is not possible to extract the precipitating antigen in adequate concentration by the standard method. In the

latter case it may be tentatively assumed either that in a few strains the antigen is present within the bacterial cell in materially smaller amounts than usual, or that, inasmuch as the antigens from different strains do not of necessity possess the same degree of stability, an

TABLE I.

	Inulin fermentation	Agglutination Serum R 867	Precipitation Serum R 862	Strains
Type I	Prompt	++++	++++	45
	"	++++	+++	11
	"	+++	++++	5
	"	+++	++ to +++	5
	"	Spontaneous	+++ to ++++	5
	"	++++	+ to ++	9
	"	+++	+	1
	"	++++	- to ±	2
	"	+++	- to ±	3
Total.....				86
Group X	Prompt	++	- to ±	2
	"	±	++	1
	"	± to +	± to +	7
	"	- to ±	- to ±	3
	"	Spontaneous	+ to ++	4
	"	"	- to ±	15
	Delayed	++	+	1
	"	+	+	2
	"	+	±	1
	"	-	-	3
	"	Spontaneous	+	1
	"	"	- to ±	8
	Negative	+	± or -	5
"	-	± or -	14	
"	Spontaneous	± or -	6	
Total.....				73

occasional one may be injured by the reagents employed. All the strains of Type I ferment both inulin and salicin promptly.

The second group comprises those organisms which agglutinate weakly or not at all in Type I antiserum at a dilution of 1:250 or higher, and which fail to yield strongly precipitating extracts by the

TABLE II.

Class	Strain	Inulin	Salicin	Agglutination: Serum R 867 Serum dilutions				Precipitation: Serum R 862 Antigen in cc.						
				1:250	1:500	1:1000	1:2000	0.2	0.1	0.025				
Type I	Q 88 E	+	+	+	+	+	+	+	+	+	+	+	+	
	RF 24 T	+	+	+	+	+	+	+	+	+	+	+	+	
	Q 166 B	+	+	+	+	+	+	+	+	+	+	+	±	
	P 5 B	+	+	+	+	+	+	+	+	+	+	+	±	
	P 86 B	+	+	+	+	+	+	+	+	±	+	+	+	±
Group X	RF 156	+	+	+	+	+	+	+	+	+	+	+	+	±
	R 9	+	+	+	+	+	+	+	+	+	+	+	+	±
	P 28 B	+	+	+	+	+	+	+	+	+	+	+	+	±
	P 71 B	-	-	-	-	-	-	-	-	?	±	±	±	±
	P 128	-	-	-	-	-	-	-	-	±	±	±	±	±
	R 1 P 16 B	-	-	-	-	-	-	-	-	±	±	±	±	±

standard method employed. In general the same correspondence appears between the results of the agglutination and precipitation tests. It is recognized that in still lower dilutions of the serum agglutination might occur, and in a few instances this possibility has been tested and found to be a fact, but such slight cross reactions in a high titer serum are not of significance from the point of view of the present study. A greater percentage of the strains in this group show a tendency to flocculate spontaneously than is the case with those of Type I. The differentiation of the strains of this group from those of Type I is distinct, but the data are insufficient to warrant the delimitation of further types at present. These strains may, however, be placed together provisionally as Group X, with the understanding that this is a frankly heterogeneous collection from which in the future it may well prove possible to separate further definite types. The members of this group may vary in their capacity to ferment inulin: in some it is well marked; in others it becomes apparent only after a lapse of several days; while in the case of many strains it is entirely lacking. The group has been further studied from the point of view of salicin fermentation. Slight precipitation occasionally occurred with the Type I serum when extracts from salicin-fermenting organisms were employed, but was never observed in the case of non-salicin fermenters. In view of the fact that all of Type I strains are salicin fermenters this cross relationship is noteworthy and suggests that the strains which do not ferment salicin are the least closely related to Type I of any studied.

In Table II is presented a composite protocol, which serves to illustrate the manner in which the data were collected and the grouping of the individual strains arrived at. It is possible that certain of the weak precipitin reactions recorded in Group X were traceable to small protein residues in the bacterial extracts. Inasmuch as only the acid-insoluble protein was removed, the solutions were obviously far from containing one antigen only.

It should be noted that in our hands Strain R 1 has consistently failed to ferment inulin unless adapted following six to eight transfers in inulin serum water, or unless faulty inulin was employed. In Small's original report this strain is designated as an inulin fermenter. This divergence may be explainable by differences in the media used.

Unless great care is observed in sterilization the inulin will be fermented by many ordinary non-fermenters. Certain lots of inulin are also easily fermented by most indifferent streptococci. It is, therefore, necessary to control carefully each new lot of media. In this work if a strain failed to ferment salicin but fermented inulin the inulin was always found to be at fault.

#### DISCUSSION.

The existence of a group of indifferent streptococci sufficiently compact to warrant separation as a distinct type suggests certain analogies to conditions within the pneumococcus family. As far as the present observations indicate, the antigen complex of these streptococci contains a substance which by the analogy of disc precipitation in immune serum may be regarded as comparable to the pneumococcus specific substance. This analogy is strengthened by the fact that the active substance is destroyed by boiling for 30 minutes with N/10 HCl, and that as this hydrolysis proceeds copper-reducing substances appear in the solution. The usually close agreement between the results of the agglutination and precipitation reactions suggests that it is this same substance which is responsible for both of the immune reactions, and that the compactness of Type I results from the presence within a great number of strains of indifferent streptococci of soluble substances identical or very similar chemically. Such a concept is comparable to that developed by Avery and Heidelberger (14) in the case of the pneumococci, and by Lancefield (15) for certain strains of *Streptococcus viridans*, but is at variance with conditions as they exist within the family of the hemolytic streptococci (16, 17). Efforts to identify the soluble substances of Type I with the species-specific soluble substance of the hemolytic streptococci have been unsuccessful. On the other hand, a few strains of *Streptococcus viridans* have been encountered which have shown agglutination in Type I serum, and from these strains it has been possible to prepare extracts which precipitate such serum with the formation of fairly compact discs. Such strains of *Streptococcus viridans*, therefore, contain antigens in common with Type I indifferent streptococci. Similar instances of cross agglutination have been reported by Birkhaug. Whether such observations have more than an academic interest is at present a matter of conjecture.



## SUMMARY AND CONCLUSIONS.

Serological study of a large number of strains of indifferent streptococci has revealed the existence of a large homogeneous group to which the designation Type I has been applied. It is recognized that members of Type I are not necessarily identical, and that further division into subtypes may be feasible. All strains of Type I ferment inulin and salicin.

The remaining strains are referred to as belonging to Group X. They are distinguished only by their failure to react strongly with Type I serum. While at present this group must be regarded as quite heterogeneous, further work may reveal the presence of other as yet undefined types now included within its limits. The organisms of this group vary in their fermentative reactions with both inulin and salicin.

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