STUDIES UPON MINUTE HEMOLYTIC STREPTOCOCCI

III. Serological Differentiation[1]

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In previous papers (Long and Bliss, 1934; Long, Bliss and Walcott, 1934) the cultural characteristics and distribution of minute hemolytic streptococci were discussed. These may be summarized as follows: Minute hemolytic streptococci produce extremely small colonies, ranging in size, in poured blood agar plates, from complete invisibility to 0.13 mm. in diameter after 18 hours and from 0.14 to 0.40 mm. after 48 hours growth. The zone of hemolysis, which is of the *beta* type, may be from $2\frac{1}{2}$ to 18 times as large as the colony. The organisms, as observed in smears, are about two-thirds the size of ordinary *beta* streptococci. They tend to lie in clumps, although moderately long chains also are present. They are amphophilic to the Gram stain. They are easily grown in blood broth and blood agar and in plain broth containing as little as 0.075 per cent glucose. Cultures in blood broth under a vaseline seal have been found to be viable after a year in the refrigerator. Most of the strains were isolated from throat cultures, some from normal individuals, more from patients suffering from nephritis and rheumatic infections. We are indebted to Dr. Rebecca C. Lancefield and to Drs. Beatrice and David Seegal for a number of strains.[2] Fifty strains, isolated from 44 individuals were studied in detail. None reduced methyl-

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[2] All of the numbered strains mentioned in this article came from these investigators, those in which the letter precedes the number (as in H59) from Dr. Lancefield and those in which the number comes first (as in 22E) from the Drs. Seegal.

ene blue, hydrolyzed sodium hippurate or fermented sorbitol. All but 5 fermented trehalose. Thirty-seven of the strains fermented salicin but not lactose or mannitol, thus resembling *Streptococcus equi*, according to Holman's classification (1916). Ten were like *Streptococcus pyogenes* in fermenting lactose and salicin but not mannitol, and 3 were like *Streptococcus subacidus* since they attacked none of these three carbohydrates.

The purpose of the present study was to determine the serological relationships of the strains of minute hemolytic streptococci in our possession.

METHODS

Lancefield's precipitin technique (1928a and b, 1933, 1934) was used both for grouping and typing the strains. Recently the types have been further established by the rapid slide agglutination method as described by Griffith (1934).

Precipitin tests

Antisera. Rabbits were immunized in the manner outlined by Lancefield (1933, 1934) for the production of sera high in carbohydrate antibody content. The vaccines were prepared from 18-hour broth cultures which were centrifuged at high speed and the sediments from which were resuspended in one-twentieth of the original volume of salt solution containing 0.2 per cent Formalin. They were stored in the refrigerator. After three days, subcultures were made to see whether the organisms were dead. Prior to using, the vaccines were diluted 20 times, that is, to their original volume.

Rabbits were given intravenous injections of 1 cc. of the diluted vaccines daily for six days and then allowed a week's rest. Five days after the last injection they were bled and the sera tested for precipitins. If the precipitin content was low the rabbits were subjected to another six-day course of 1 cc. injections. This cycle was repeated until the sera gave good reactions with the homologous antigens, at which time the rabbits were exsanguinated and the sera collected. Theoretically, one should use a heterologous strain of the same group in testing for group anti-

bodies but this is impossible when one is dealing with a collection of unidentified strains.

Lancefield (1934) states that, where group B strains are concerned, the sera of rabbits immunized with one or two courses of injections are usually high in group ("C" substance) antibody content but that if the injections are continued this tends to disappear or to be masked by increasing amounts of type-specific antibody. The majority of our sera were collected after two courses of injections but some gave excellent reactions after one series and were harvested then, while a few required three, or even four, courses.

Antigens. The antigens, also, were prepared according to Lancefield's directions. The streptococci from a 250 cc. broth culture were centrifuged out and resuspended in 5 cc. of 0.85 per cent salt solution. The suspension was acidified by the addition of 0.25 cc. of normal HCl, placed in boiling water for ten minutes, cooled in running water and centrifuged till clear. The supernatant fluid was neutralized to phenol red with 2 N NaOH and was kept in the refrigerator overnight. The following morning the flocculent precipitate which was usually, but not invariably, present was removed by centrifugation. The antigens were then ready for use.

Lancefield pointed out that these HCl extracts contain a number of antigenic substances and she showed that some purification could be effected by precipitation with 3 volumes of 95 per cent alcohol. In both group A and group B the group or "C" antigen remained in the supernatant fluid while the type-specific fraction precipitated out. In many of our experiments, therefore, the crude antigen was treated with 3 volumes of alcohol in the hope that a greatly purified group specific antigen would be obtained. After the addition of the alcohol the antigens were kept overnight in the refrigerator. The precipitate was removed by centrifugation and the supernatant fluid evaporated to dryness on a steam bath. The dry residue was taken up in the original volume of 0.85 per cent salt solution and, after thorough mixing, any insoluble material was centrifuged out.

Tests. Three concentrations of antigen were used in the tests,

undiluted, diluted 1:4 and 1:16. To 0.4 cc. of each dilution of antigen 0.2 cc. of serum was added, care being taken to layer it in the tube. The tests were left at room temperature for 10 to 20 minutes and examined for ring formation. They were then shaken and placed in the 37°C. water bath for 2 hours and read again. Final readings were made after they had stood in the refrigerator overnight.

Agglutinin tests

Antigens. The organisms, grown in broth for 18 to 20 hours at 37°C., were centrifuged out of the culture medium and resuspended in one-fiftieth of the original amount of broth. Most of the minute hemolytic streptococci grow diffusely in broth; those that grow granularly, however, can be got into a sufficiently smooth state for the slide agglutination test by pipetting the suspensions back and forth, particularly if the pipette is held so firmly against the bottom of the tube that considerable suction and pressure are required to force the material up and down (Eagles, 1926).

Antisera. The sera were the same as those used in the precipitin tests. Here they were used in a 1:5 dilution.

Tests. A clean slide was marked off in squares with a china marking pencil and one drop of the suspension from a capillary pipette was placed in each square. To one drop, which served as the suspension control, was added a small loopful of salt solution; to the others was added a small loopful of the 1:5 dilutions of the various sera. The serum and suspension were mixed thoroughly with the loop and, after the complete series was set up, the whole slide was rocked gently by hand. Agglutination when present was easily visible to the unaided eye. It occurred in from a few seconds to three minutes after the mixture was made.

Tryptic digestion

Several of the HCl extracts for the precipitin test were subjected to digestion with trypsin. The following method was employed:

To 5 cc. of the extract at pH 7.6 were added 100 mgm. of Fairchild's trypsin. After thorough shaking the mixture was incu-

bated at 37°C. for two hours. The activity of the trypsin was then destroyed by heating in boiling water for 10 minutes and the suspension clarified by centrifugation. Controls consisted of antigen subjected to the same procedure except that the activity of the trypsin was destroyed immediately after its addition.

RESULTS

Grouping

Preliminary tests with sera from rabbits immunized against ten strains of minute hemolytic streptococci and their corresponding antigens seemed to show (Bliss and Long, 1935) that there were at least three different groups of these organisms (table 1). One strain from each of these apparent groups was therefore chosen as the type strain and was used to immunize four rabbits. The sera from each set of four rabbits were pooled. Tests were set up with these sera against both crude HCl extracts and the alcohol-supernatant fluids of fifty-four strains of minute streptococci. The results are shown in table 2. Fourteen strains reacted predominantly with For serum, thirty-four with Hav and six with Ruf. Two of the latter, however, failed to react with Ruf serum when the alcohol-supernatant fluids were used, suggesting that the reactions obtained with the crude antigens were cross reactions and that therefore these two strains in reality belonged to a fourth group for which no serum had been prepared.

Cross reactions, as can be seen in the table, were numerous. Many of these were eliminated when purified antigens were used but with seven strains (cf. S and Gre in the table) they persisted in the tests with alcohol-supernatant fluids.

Because of the prevalence of cross reactions, which should not obtain between groups, Dr. Lancefield, whom we consulted frequently about these tests and who has been of inestimable help to us throughout the study, believed that we must be dealing with type rather than group differences,—that the main reactions were type reactions while the "cross reactions" possibly represented group relationships. She suggested that our sera might not be group-specific and, in view of this doubt, that it would be advisable to try to deprive some of the strains of their type specificity

TABLE 1

Preliminary precipitin tests with sera made from 10 strains of minute streptococci and their corresponding antigens, showing apparent existence of three groups

ANTIGENS	SERA									
	For	Whi	S	Holc	Hav	Ter	N	22E	W	Ruf
For	++++	±	+++ D	+++ D	+	−	−	++	+	+
	++++	++	+++ D	+++ D	−	−	−	−	+	−
	++++ D	++	+++ D	++++ D	−	−	−	−	++	−
Whi	++	+++	++++	+++ D	±	−	−	++	+	+
	+++ D	+++	++++ D	++++ D	+	−	+	++	+	+
	++++ BD	++++ D	++++ D	++++ BD	±	ST	+	+	+	−
S	+++	++++	++++	+++ D	±	−	±	++	++	++
	+++ D	+++		++++ D	++	±	++	+	+	+
	++++ BD	+++		+++ BD	++	−	−	+	+	−
Holc	++++	+++	++++	+++ D	+	−	++	++	+++	+++
	++++	+++	++++	++++ D	−	−	+	+	+	+
	++++ BD	+++	+++ D	++++ BD	−	−	−	+	+	−
Hav	+	++	++ D	+	++++ D	++++	++++ D	++++ D	++++ D	+
	−	++	++ D	+	++++	++++	++++ D	++++ D	++++ D	−
	−	++	+	++ Gr	+++	+++	+++	+++	+++	−
Ter	+	+	+	±	++++ D	++++	++++ D	++++	++++ D	+
	−	++	+	±	++++ D	++++	++++	++++ D	++++ D	−
		−	+	±	+++	+++		++++	++++ D	−
N	+	+	++	+	++++ D	++	++++	+++	++++ D	++
	−	++	++	+	++++ D	++++ D	++++ D	++++ D	++++ D	+
	−	++	++ D	+ Gr	++++	++++ D	++++	++++ D	++++ D	−
W	+++	+	++	++	++++ D	++	+++	++++	++++ D	++++
	+++	++	++	+	++++ D	++++	++++	++++	++++ D	++
	+++ BD	++	+	++	++++	++++	++++	++++	++++ D	+
Ruf	+++	++	++	++	++	±	++	+++	+++	++++
	+	±	+	+	±	−	+	+++		++
	−	−	−	±	−	−	−	+	+	+

Twenty-hour readings—3 dilutions.
D = Disc, BD = Broken Disc, Gr = Granular, T = Turbid, ST = Slightly Turbid.

by cultivating them in immune serum and to use these altered strains for the immunization of rabbits.

This was done but the results were so confusing that they will not be described here. Suffice it to say that group-specific antisera were not acquired by this method.

In the meantime Dr. Lancefield reported to us that she had succeeded in getting an anti "C" serum with a strain, H59, which she had isolated and that nearly all of the strains which we had sent her were precipitated by it. The strains included a number each of our For and Hav "groups." She concluded that they were all members of one group and suggested that this group be designated by the letter F. At the same time she found that three other members of our Hav "group," including Hav itself and Mad and Ter were not precipitated by this serum but did come down in a serum prepared against a strain from group G, in which hitherto only large hemolytic streptococci had been placed. Hav, Mad, and Ter, then, differed in respect to "C" substance from the other strains to which they had previously seemed to be related. As will be shown when the agglutination reactions are presented this bond between Hav, etc. and the other strains lies in their having a common type specific antigen. A similar immunological relationship between heterogeneous organisms was shown by Avery, Heidelberger and Goebel (1925) to exist between pneumococcoccus type II and a strain of Friedländer's bacillus.

Dr. Lancefield kindly sent us her group-specific serum and the strain, H59, with which it had been prepared and also the group G serum and group G strains. After preliminary tests with the sera, in which Dr. Lancefield's observations were confirmed, rabbits were immunized against the strains and fifty-five of our cultures were tested with the new sera. The results are shown in table 3. Forty-nine of the fifty-five strains reacted with the group F—H59 serum and ten with the group G—H13 (extracts from 4 strains precipitated in both sera). The reactions with the H59 serum ranged from ± to +++ with disc. Those with H13 were weaker, but, though the sera made with this strain were always very poor, they served to distinguish between the group F

TABLE 2

Examples of the different kinds of reactions given by crude and alcoholic extract antigens of minute streptococci in three antisera

ANTIGENS		ANTISERA									NUMBER OF STRAINS GIVING SIMILAR REACTIONS
		For			Hav			Ruf			
		Ring	2 hours	20 hours	Ring	2 hours	20 hours	Ring	2 hours	20 hours	
For	Crude	++++	++ BD	++ D	−	−	±	+	T	++	9
		++++	+++ BD	+++ D	−	−	±	±	+	+++	
		++++	+++ BD	+++ D	−	−	±	−	−	+	
	Alcohol supernatant	++	+	++ D	−	−	−	−	−	ST to +	
		+	T	++ BD	−	−	−	−	−	−	
		±	−	T	−	−	−	−	−	−	
S	Crude	+++	±	+	±	−	±	+	+++	++++	5
		+++	+++	+++ D	±	−	+	+	+	++	
		+++	+++	+++ D	±	−	++ BD	±	ST	±	
	Alcohol supernatant	±++	±	+	±	−	+	−	−	−	
		+++	++	++ D	±	−	+	−	−	−	
		+++	+++ BD	+++ D	±	−	++ BD	−	−	−	
Gre	Crude	±	−	±	++++	+	+	+	+	++	2
		±	−	±	+++	++ BD	++ BD	±	+	++	
		±	−	+	+++	++ D	+++ D	−	−	±	
	Alcohol supernatant	−	−	−	+++	−	−	−	−	ST	
		−	−	+ BD	+++	++	++ BD	−	−	−	
		−	−	+ BD	+++	++ D	+++ D	−	−	−	

Hav	Crude	−	−	+	++++	++	++ D	+	+	+	31
		−	−	+	++++	+++ D	+++ D	−	ST	+	
		−	−	+	++++	+++ D	++++ D	−	−	±	
	Alcohol supernatant	−	−	−	++++	++ D	++ D	−	−	− to +	
		−	−	−	++++	+++ D	+++ D	−	−	−	
		−	−	−	+++	+++ D	++++ D	−	−	−	
Mad	Crude and alcohol supernatant	−	−	−	++++	++	++ D	−	−	−	1
		−	−	−	++++	++ D	+++ D	−	−	−	
		−	−	−	+++	+++ BD	++++ D	−	−	−	
Ruf	Crude	−	−	−	−	−	−	++	T	+++	4
		−	−	+	−	−	+	++	+++	++++	
		−	−	+	−	−	±	++	++	+++	
	Alcohol supernatant	−	−	−	−	−	−	−	T	+	
		−	−	−	−	−	−	−	−	−	
		−	−	−	−	−	−	−	−	−	
She	Crude	±	ST	+	−	−	T	+	T	+	2
		−	ST	+	−	−	ST	+	+	++	
		−	ST	+	−	−	−	−	−	+	
	Alcohol supernatant	−	−	−	−	−		−	−	−	
		−	−	−	−	−		−	−	−	
		−	−	−	−	−		−	−	−	

and group G strains, since four of the six strains which did not react with H59 gave disc precipitates with H13. Two strains failed on repeated tests to react with H59 and gave only ± reactions with H13. Of these, one—H93—had been found by Dr.

TABLE 3

Precipitin reactions in anti "C" sera

ANTIGENS	SERA		GROUP	ANTIGENS	SERA		GROUP
	Group F H59	Group G H13			Group F H59	Group G H13	
Bay	++ D	−	F	Hall	+ D	−	F
Che	++ D	−	F	Sma	+	−	F
Gre	++ D	−	F	Ant	++ BD	−	F
Hav	++ D	−	F	D	++ BD	−	F
Hyd	++ D	−	F	For	++ BD	−	F
Jon	++ D	−	F	Holc	++ BD	−	F
Kla	++ D	−	F	Pai	−	±	F?
Merr	++ D	−	F	Rea	++ D	±	F
Mers	++ D	−	F	S	++ D	−	F
Mye	++ D	−	F	Stu	+ BD	−	F
N	±	±	F?	40E	+	−	F
Pau	++ D	−	F	84E	+	−	F
Str	++ D	−	F	Arr	+ D	−	F
Tat	++ D	−	F	Kra	+	−	F
Ver	++ D	−	F	Neu	+ D	−	F
W	++ BD	−	F	Dei	±	−	F?
MHW	++ BD	−	F	Ham	±	±	F?
Win	+ D	−	F	Moo	+ BD	−	F
Wri	++ D	−	F	Ruf	+	−	F
22E	+	−	F	She	±	−	F?
38E	+	−	F	86E	+ BD	−	F
45E	++ BD	±	F	H59	+++ D	−	F
65F	++ BD	−	F				
95E	++ D	−	F	Hav	−	+ D	G
101F	++ BD	−	F	Mad	−	+ D	G
103H	++ BD	−	F	Pen	−	+ D	G
106F	+	−	F	Ter	−	+ BD	G
Fis	++ BD	−	F	H93	−	±	G?

Lancefield to belong to group G. The other, Pai, behaves in other respects like For and presumably should be grouped with it. Four strains gave negligible or equal reactions in the two sera. One is strain N which has been placed in group F but might equally well go in group G except that it reacts more strongly with

TABLE 4

Agglutination reactions by "direct slide technique"

ANTIGENS	SERA						ANALYSIS	
	Group F				Group G			
	MHW	For	Ruf	H59	Hav	H13	Group	Type
Bay	+	−	−	−	+++	−	F	1
Cne	+++	−	−	−	+++	−	F	1
Gre	+++	−	−	−	+++	−	F	1
Hav	++	−	−	−	+	−	F	1
Hyd	+++	−	−	−	+++	−	F	1
Jon	+++	−	−	−	+++	−	F	1
Kla	+++	−	−	−	+++	−	F	1
Merr	+++	−	−	−	+++	−	F	1
Mers	++++	−	−	−	++++	−	F	1
Mye	+++	−	−	−	++	−	F	1
N	+++	−	−	−	+++	−	?	1
Pau	+++	−	−	−	+++	−	F	1
Str	++++	−	−	−	+++	−	F	1
Tat	+++	−	−	−	+++	−	F	1
Ver	+++	−	−	−	+++	−	F	1
W	+++	−	−	−	+++	−	F	1
MHW	+++	−	−	−	++++	−	F	1
Win	+++	−	−	−	++	−	F	1
Wri	+++	−	−	−	+++	−	F	1
22E	++++	−	−	−	++++	−	F	1
38E	+++	−	−	−	++++	−	F	1
45E	+++	−	−	−	++++	−	F	1
65F	+++	−	−	−	++	−	F	1
95E	+++	−	−	−	+++	−	F	1
101F	++++	−	−	−	++++	−	F	1
103H	++++	−	−	−	++++	−	F	1
106F	+++	−	−	−	+++	−	F	1
Fis	+++	−	−	−	+	−	F	1
Holl	+++	−	−	−	+++	−	F	1
Sma	+++	−	−	−	+++	−	F	1
Ant	−	+	−	−	−	−	F	2
D	−	+	−	−	−	−	F	2
For	−	++	−	−	−	−	F	2
Holc	−	++	−	−	−	−	F	2
Pai	−	+++	−	−	−	−	?	2
Rea	−	+	−	−	−	−	F	2
S	−	+	−	−	−	−	F	2
Stu	−	+	−	−	−	−	F	2
40E	−	+	−	−	−	−	F	2

TABLE 4—*Concluded*

ANTIGENS	SERA						ANALYSIS	
	Group F				Group G			
	MHW	For	Ruf	H59	Hav	H13	Group	Type
84E	−	++	−	−	−	−	F	2
Arr	−	+	−	−	−	−	F	2
Kra	−	+	−	−	−	−	F	2
Neu	−	++	−	−	−	−	F	2
Dei	−	−	+++	−	−	−	?	3
Ham	−	−	++	−	−	−	?	3
Moo	−	−	+++	−	−	−	F	3
Ruf	−	−	++	−	−	−	F	3
She	−	−	+++	−	−	−	?	3
86E	−	−	+++	−	−	−	F	3
H59	−	−	−	+++	−	−	F	4
Hav	+++	−	−	−	+++	−	G	1
Mad	+++	−	−	−	+++	−	G	1
Pen	+++	−	−	−	+++	−	G	1
Ter	+++	−	−	−	+++	−	G	1
H93	+++	−	−	−	+++	−	?	1

Ruf serum than do any of the group G strains. The other three were previously classed with Ruf.

In summary, fifty of the fifty-five strains which were studied fall into one group which is to be called group F. The remaining five belong to group G.

Typing

The readiness with which the minute streptococci produce type antibodies stood us in good stead when it came to typing them. During the process of grouping, the outlines of the type relationships were all too clearly visible. When Griffith's slide agglutination technique was applied to the strains it was seen that the agglutination and precipitin reactions paralleled each other. The results of the agglutination reactions are shown in table 4. They were absolutely clear cut.[3] Four types have so far been demon-

[3] Except for one strain, no cross reactions were seen. The excepted strain, Bul, which agglutinated in For, MHW and Hav sera was isolated just as this work

strated in group F, one like For, one like Ruf, one, represented only by strain H59 and one like MHW which comprises the largest number of strains and which is identical with the type in group G to which Hav belongs. It has been decided, in conjunction with Dr. Lancefield, to call this last type type I in both groups F and G. Type For, then is Type 2; Ruf, 3 and H59, 4.

DISCUSSION

The important point in the grouping of streptococci by Lancefield's precipitin method is the use of sera high in group or "C" antibody content. The difficulties encountered by us in the grouping of the minute hemolytic streptococci may be ascribed to two factors. First, all but one of the strains of minute streptococci were so extremely type-specific that antisera prepared against them contained no group antibody, and second, we relied upon the use of the correct antigen rather than of the correct antiserum. In doing so we exposed ourselves to two hazards. First, that the "correct antigen" might fail to react with an incorrect serum and second, that we might not recognize the "correct antigen." The latter was probably our main pitfall. Lancefield had shown that with both the group A and group B hemolytic streptococci the type and group specific antigens could be separated by precipitation with alcohol. With the group A streptococci (1928a), the type specific "M" fraction was apparently a protein and could be precipitated from the crude antigen with 3 volumes of alcohol, leaving in solution the "C" or group antigen. With the group B streptococci (1934), although the type specific fraction proved to be a carbohydrate, the same held true. The "S" or type substance again was precipitable with 2 to 3 volumes of alcohol. Therefore, at the start of the work with the minute streptococci, it was assumed that the alcoholic supernatant fluids contained the group substance in a purified state and that precipitin reactions obtained with this material were group reactions. That this was not the case was shown when a good anti "C"

was being brought to a close and there was no opportunity to test it for agglutinin absorption. At first it was thought that perhaps the culture was a mixture of two types of minutes but strains from nine colonies behaved alike and like the parent culture. Its strongest precipitin reaction was with For serum.

TABLE 5

Precipitin reactions with four antigens and their alcoholic fractions

ANTIGENS		SERA					
		Type			Group		
Ant.	Crude	++	±	T	+	+	+
		++	+	+ BD	+	+	+
		++	++	++ D	+	−	+
	Alcohol supernatant	+	−	−	+	−	−
		+	−	+ BD	+	−	±
		−	−	++ D	−	−	+
	Alcohol precipitate	++	ST	+	+	ST	+
		++	T	+	+	±	+
		+	T	++ BD	−	−	±
D	Crude	+	−	−	+	+	+
		+	±	+	+	±	+
		+	++	++ D	+	ST	+
	Alcohol supernatant	+	−	−	+	−	ST
		+	± BD	+ D	+	−	±
		+	+	++ BD	+	−	+
	Alcohol precipitate	+	++	+ BD	+	+	+
		+	T	±	+	+	+
		0	0	0	−	T	+
Mye	Crude	0	0	0	+	T	+
		++	+	++	+	T	+
		++	+	++	±	−	+
	Alcohol supernatant	++	+	T	−	−	+
		++	+	++ D	−	−	+
		+	+	+++ D	−	−	+
	Alcohol precipitate	+	++	++	+	+	+
		+	ST	+	±	±	+
		−	−	±	−	−	±
W	Crude	+++	+++	+++ BD	+	+	+
		++	+++	+++ BD	+	±	+
		+	+++	+++ BD	−	−	±
	Alcohol supernatant	++	+ BD	+ BD	+	−	±
		++	++ BD	++ D	+	−	+ BD
		+	+ BD	+++ D	±	−	ST
	Alcohol precipitate	+++	+++ BD	+++ BD	+	−	+
		++	++	+++	+	ST	+
		+	−	+	−	−	+

serum was at last acquired and the conclusion was perforce arrived at that a part at least of the type-specific fraction of the minute streptococci resided in the alcoholic supernatant. A preliminary attempt has been made to analyse or separate the two fractions. One experiment, in which the alcoholic supernatants, precipitates and crude antigens of four strains were tested against their type and group sera, seemed to show (table 5) that

TABLE 6

Precipitin reactions of antigens before and after digestion with trypsin

ANTIGENS		SERA	
		Type	Group
40E	Digested with trypsin	++ D	+ BD
		+++ D	+ D
		++ BD	+ BD
	Not digested	+	+
		+++ D	+ D
		+++ BD	+
Che	Digested with trypsin	+	+
		++ D	+ D
		++ D	+ D
	Not digested	++ D	+
		+++ D	+ D
		++++ D	++ D
Mye	Digested with trypsin	++ D	+ D
		++++ D	++ D
		++++ D	+ BD
	Not digested	++ BD	+
		+++ D	+ D
		++++ D	++ D

part of each antigenic substance was to be found in each alcoholic fraction. Another experiment, in which tryptic digestion was tried, indicated (table 6) that both substances might be carbohydrate since neither lost in potency by this treatment.

Another obstacle to the ready interpretation of the precipitin reactions of the minute hemolytic streptococci has been the prevalence of cross reactions. Some of these, no doubt, are attribut-

able to rudimentary group reactions in the presence of type antigen and antibody. Others cannot be explained in this way. For instance, the disc precipitates formed by the strains illustrated by S (in table 2) in Hav serum cannot be explained on this basis, since the five antigens differed in both group and type from the strain against which the serum was produced. These reactions did not occur on single occasions but were observed repeatedly. Then what is the basis for the precipitations in Ruf serum? The group G strains rarely reacted with this serum, those in group F did so constantly, and yet, if these reactions marked a group differentiation, why were they so largely eliminated by the use of

TABLE 7

SOURCE	NUMBER OF STRAINS	F				G
		1	2	3	4	1
Nephritis	16	10	6			
Rheumatic fever	3	3				
Infections caused by minute streptococci	5	4	1			
Other diseases	14	6	4	2	1	1
Normals	13	6	1	3		3
Unknown	4	13	1	1		1
Total	55	30	13	6	1	5

alcoholic extracts? The antigens of the Ruf strains behave quite differently from those of other strains. As has been said, no strain, heterologous or homologous, gave a disc precipitate with Ruf serum, though frequently the reactions were strong. In addition to this, however, only two of the Ruf strains produced discs with the anti "C" serum of strain H59. Were it not for these two strains we should be inclined to continue in our belief that the Ruf strains constitute a third group of minute streptococci. As it is (and since all the Ruf strains have the same type antigen, as shown by the agglutination reaction), they will be assigned to group F until further information is available.

Finally, there were, beside the Ruf strains, those three others

which gave negligible reactions in both the group F and group G anti "C" sera. Behaviour of this sort is explicable on the ground that such strains possess little or no group specific substance in their antigenic complex.

When the serological classification of the minute hemolytic streptococci is compared with the biological "grouping" reported in an earlier paper (Long and Bliss, 1934) no relation between the two is demonstrable, except that no members of types 1, 2 or 4 of group F belong to the second biological group, which contained the ten pyogenes-like strains.

When the source of the strains used in the present study, is compared with their serological classification (table 7) it is found that the sixteen strains from patients with glomerular nephritis, the three strains from rheumatic fever patients and the five strains which apparently were the primary cause of disease[4] all belong to type 1 or 2 of group F.[5]

SUMMARY

Fifty-five strains of minute beta hemolytic streptococci have been serologically differentiated. Fifty of the strains fall into one group which has been assigned the letter F by Lancefield. No ordinary beta hemolytic streptococci have been shown to belong to this group up to the present time. Five strains belong to group G, Lancefield. The previously isolated members of this group were ordinary beta hemolytic streptococci.

The fifty group F strains may be divided into four types. There are thirty strains in type 1, thirteen in type 2, six in type 3 and one in type 4.

The five strains of minute streptococci which fall into group G all belong to one type. The type antigen of these strains is identical with that of the members of group F, type 1.

It is difficult to group the minute streptococci because of their extreme type specificity.

[4] Paper in press by Long and Bliss.

[5] Since this paper was written a group F, type 3 strain has been recovered as the primary pathogenic agent in a case of acute pansinusitis.

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