STUDIES ON NON-HEMOLYTIC STREPTOCOCCI ISOLATED FROM THE RESPIRATORY TRACT OF MAN

The Antigenic Basis for Type Specific Reactions with Streptococcus salivarius and Non-Levan-Forming Streptococci

By FRANK L. HORSFALL, Jr., M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

(Received for publication, October 30, 1950)

Efforts to determine the relationship of non-hemolytic streptococci to acute respiratory infections are seriously hampered by a lack of adequate information on the antigenic constitution of these microorganisms (1). In the absence of such information, precise identification of a particular strain is difficult and serological classification of a number of strains has uncertain significance. That some species of non-hemolytic streptococci are associated with respiratory infections appears evident from the results of studies on streptococcus MG (2–4) in primary atypical pneumonia (5, 6). That other species of non-hemolytic streptococci are causally related to an even more serious disease in man is evident from investigations on *Streptococcus sanguis* (s.b.e.) in subacute bacterial endocarditis (7, 8).

Numerous investigators have employed serological procedures for the classification of non-hemolytic streptococci. The most extensive studies are those of Birkhaug (9), Hitchcock (10), Solowey (11), and Sherman, Niven, and Smiley (1). The available evidence indicates that group specific antigens analogous to those of beta hemolytic streptococci (12) are not present. However, evidence for the presence of serological types has been obtained repeatedly (1). The nature of the antigenic constituent which is responsible for the type specific reactions appears not to have been determined except in the case of streptococcus MG (3) and *Streptococcus salivarius* type I (4).

Among the numerous species of non-hemolytic streptococci which inhabit the upper respiratory tract of normal persons, that about which most is known as to antigenic structure is streptococcus MG. As was shown previously (3) this microorganism elaborates a capsular polysaccharide which confers upon it type specific immunological properties; the antigens present in the body of the bacterial cell are distinct from and unrelated to the capsular substance. The present view is that the antigenic structure of streptococcus MG is closely analogous to that of type specific pneumococci and that the various serological reactions obtained with these different bacterial species are attributable to similar substances similarly distributed about the bacterial cell.

Among the non-hemolytic streptococci present in the throat of human beings, *Str. salivarius* occupies a prominent place and is recoverable from as many as 55 per cent of normal persons (1). Distinct serological types of *Str. salivarius* were demonstrated clearly by Sherman, Niven, and Smiley (1) who separated the species into types I, II, and an indeterminable number of other types. In this laboratory it was shown (4) that the type specific serological properties of type I *Str. salivarius* are dependent upon the presence of a capsular polysaccharide closely analogous to that of streptococcus MG. An immunological relationship between these two different microorganisms was demonstrated and shown to result from certain similarities in their capsular substances. Preliminary studies (4) raised the possibility that type II *Str. salivarius* also elaborates a capsular polysaccharide of similar immunological significance.

Much attention has been directed toward the immunologically active polysaccharide (levan) which is synthesized by *Str. salivarius* in the presence of sucrose (13) as not by streptococcus MG (2). It has been shown that an enzyme, separable from the bacterial cell, can synthesize levan from sucrose *in vitro* (14) and that levans formed in the presence of sucrose by various members of the genus *Bacillus* possess serological properties similar to those of the levan produced by *Str. salivarius* (15, 16). No clear distinction seems to have been made between the type specific capsular polysaccharides of *Str. salivarius* and those polysaccharides which are formed enzymatically by these bacteria in the presence of an appropriate substrate (sucrose).

It is the purpose of this paper to show that, like type I *Str. salivarius*, type II *Str. salivarius* elaborates a capsular polysaccharide which endows it with type specific immunological properties and that the capsular polysaccharides derived from the two types are serologically distinct. It will be shown also that the antigens present in the cell bodies of non-encapsulated R variants obtained from the two types are not identical although somewhat related immunologically. In addition, it will be demonstrated that the levans formed from sucrose by the two types of the microorganism, whether encapsulated or devoid of capsules, are immunologically indistinguishable and are unrelated to either of the two capsular polysaccharides or to antigens in the cell bodies. Evidence indicating that various non-levan-forming streptococci also give type specific immunological reactions will be presented.

Materials and Methods

Str. salivarius.—*Str. salivarius* type I, S31A, and *Str. salivarius* type II, S30D, which are the same representative strains used in earlier studies (3, 4) were kindly supplied by Dr. J. M. Sherman, Cornell University, Ithaca.

R Variants.—Repeated subcultures in broth containing 50 per cent homologous immune rabbit serum were carried out in a manner identical with that employed to obtain non-encapsulated R variants from streptococcus MG (3). After only 14 serial transfers under these conditions, marked alterations in cultural and immunological properties of both types of *Str. salivarius* were observed, but on serial transfer in the absence of immune serum the variant strains eventually reverted to the original encapsulated or S form. However, after 28 transfers in the presence of homologous immune serum, the alterations mentioned above

persisted and reversion to the encapsulated S form did not occur despite numerous subcultures in the absence of serum. R variants from these latter series were used in the present study. It should be emphasized that, as was found with streptococcus MG (3), R variants of *Str. salivarius* could not be distinguished from encapsulated (S) cells on the basis of colony morphology although they could be differentiated readily when grown in liquid medium.

Streptococcal Suspensions.—For agglutination reactions as well as for the immunization of rabbits, streptococcal suspensions were prepared exactly as described previously (3): 18-hour Todd-Hewitt broth cultures were heat-killed and the bacterial cells collected by centrifugation after which they were washed 3 times with buffered saline. The washed cells were resuspended in buffered saline to approximately 1/5 the original volume which yielded a turbidity corresponding to a reading of approximately 225 with the Klett-Summerson photoelectric colorimeter. When it was desired to grow the streptococci in the presence of sucrose, 5 per cent of reagent grade sucrose was added to the medium.

Capsular Polysaccharides.—These were separated and purified from large quantities of Todd-Hewitt *broth* culture of the desired streptococcus by the water extraction method (B) described in detail in a previous communication (3). The yields and the chemical properties of the preparations obtained from either type I or type II organisms corresponded closely with those of earlier preparations of type I *Str. salivarius* capsular polysaccharide (4). It should be emphasized that sucrose was not present in cultures used for a source of capsular polysaccharide.

Levan.—Levan was prepared enzymatically through the action of cell-free filtrates of Todd-Hewitt *broth* cultures of the desired streptococcus on sterile 5 per cent sucrose: 1/10 to 1/20 volume of sterile culture filtrate was added to 5 per cent sucrose solution in water. A few drops of chloroform were added and the mixture was held for 48 to 72 hours at 37°C. The polysaccharide was precipitated by the addition of 4 volumes of ethyl alcohol saturated with sodium acetate. The precipitate was washed with 80 per cent ethyl alcohol on a sintered glass filter and then dissolved in a small quantity of water. The polysaccharide was reprecipitated with alcohol and washed in a similar manner a second time. It was then shaken with a mixture of chloroform and amyl alcohol (17) repeatedly until free of protein. Thereafter, it was again precipitated and washed with alcohol, then washed with ether and dried in vacuum. The method was similar to those employed by others (15, 18, 19).

Immune Sera.—Rabbits were immunized with repeated intravenous injections of washed streptococcal suspensions prepared as described above. The schedule of the injections and the amounts given were identical with those described in a previous communication (3). Sera were obtained 4 to 5 weeks after beginning immunization.

EXPERIMENTAL

The antigenic constitution of encapsulated (S) and non-encapsulated (R) variants of type I and II *Str. salivarius* was investigated by a variety of immunological procedures. The experimental program was similar in scope to that used previously for the investigation of the antigenic constitution of streptococcus MG (3). Because of the fact that *Str. salivarius* forms levan in the presence of sucrose, it was necessary to exclude this disaccharide from all cultures of the bacteria in experiments concerned with the antigenic makeup of the microorganisms themselves. The results of these experiments are considered in the first section of the paper. In the second section the results of experiments with bacteria grown in the presence of sucrose and with enzymatically synthesized levan are presented.

Cross-Agglutination Reactions with S and R Forms.—A number of cross-agglutination reactions were carried out with *Str. salivarius* types I and II, as well as the R variants derived from each microorganism. The technique employed was as follows:—

Heat-killed streptococcal suspensions obtained from broth cultures containing no sucrose were prepared as described above and used both for the immunization of rabbits and in agglutination tests with immune sera. The procedure was identical with that employed in previous studies (3, 4) except that the sera were heated at 56°C. for 30 minutes before use

TABLE I

Results of Cross-Agglutination Tests with Str. salivarius Type I, Type II, and R Variants Derived from Each

Rabbit serum	Streptococcal suspension	Serum dilution*								
Immune *versus*		10	20	40	80	160	320	640	1280	2560
Type I (S)	Type I (S)	4‡	4	4	4	4	4	3	2	0
" " "	" " R var.	+§	+	+	+	+	+	+	+	±
" " "	Type II (S)	3	2	1	0	0	0	0	0	0
" " "	" " R var.	+	+	+	+	±	0	0	0	0
Type II (S)	Type I (S)	2	±	0	0	0	0	0	0	0
" " "	" " R var.	+	+	+	+	±	0	0	0	0
" " "	Type II (S)	4	4	4	4	4	4	3	2	0
" " "	" " R var.	+	+	+	+	+	+	+	+	±
R variant (type I)	Type I (S)	4	4	4	2	1	0	0	0	0
" " " "	" " R var.	+	+	+	+	+	+	+	+	±
" " " "	Type II (S)	2	0	0	0	0	0	0	0	0
" " " "	" " R var.	+	+	+	±	0	0	0	0	0

* Expressed as the reciprocal.

‡ 1 to 4 = degree of firm disc-like agglutination of S type.

§ ± to + = degree of fragile agglutination of R type.

and the reaction temperature was 22 to 25°C. Sera from a number of rabbits immunized with the desired strain were each tested against a number of streptococcal suspensions prepared from each type of encapsulated (S) cell as well as from each R variant. In many cross-agglutination experiments all of the variables relative to the variety of the immune serum and the variety of the streptococcal suspension were tested simultaneously.

The mean results obtained in a considerable number of cross-agglutination tests with type I and II and their R variants are shown in Table I. With homologous immune serum both types I and II cells gave firm disc-like agglutination of the S type closely similar to that which is obtained under analogous conditions with streptococcus MG (3) or with type specific pneumococci. Cross-reactions between types I and II were minimal but definite; with immune

serum of the heterologous type the agglutination titer was on the average only 1 per cent of that obtained with homologous immune serum. In no instance did either R variant yield agglutination of the S type irrespective of the origin of the immune serum employed. In all cases the agglutinated R cells were easily dispersed and the soft, fluffy and fragile type of agglutination with such cells was closely similar to that obtained with R variants of streptococcus MG (3) or pneumococci. Cross-reactions between the R variants of types I and II, respectively, were more marked than between the corresponding S cells. With immune serum against the heterologous type of S cells, the agglutination titer with R variants was on the average only 6 per cent of that obtained with immune serum against the homologous type of S cells. Immune

TABLE II

Results of Cross-Precipitation Tests with Culture Filtrates of Str. salivarius Type I, Type II, and R Variants Derived from Each

Rabbit serum Immune *versus*	Culture filtrates	Precipitation
Type I (S)	Type I (S)	+++
" " "	" " R var.	0
" " "	Type II (S)	0
Type II (S)	Type I (S)	0
" " "	Type II (S)	+++
" " "	" " R var.	0

sera against R variants gave, in most instances, some agglutination with the homologous type S cell. In general, the anti-S titers obtained with such sera were less than 6 per cent of the homologous titers of anti-S cell sera.

Cross-Precipitation Reactions with Culture Filtrates of S and R Forms.— Sterile filtrates of broth cultures, containing no sucrose, of type I, type II, or either of their R variants, were tested for their capacity to yield precipitates when mixed with homologous or heterologous immune serum.

The capillary precipitin technique (20) was employed and the procedure was in all respects identical with that used previously in studies on streptococcus MG (3). As with the cross-agglutination reactions described above, a number of sera as well as a number of culture filtrates obtained from each S cell or R variant were used. In every experiment a number of variables were tested simultaneously.

The mean results of numerous cross-precipitation tests with culture filtrates of types I and II as well as their R variants are shown in Table II. In every instance filtrates of S cell cultures gave precipitates only with homologous antiserum and in no case did filtrates of R variant cultures give precipitates with either homologous or heterologous immune serum. It appears evident

that a type specific substance is present in the filtrates of cultures of S cells but is not present in similar preparations obtained from R variants derived from such microorganisms.

Cross-Capsular Swelling Reactions with S and R Forms.—A summary of the results of numerous cross-capsular swelling tests with types I, II, and their R variants is presented in Table III. The technique employed was identical with that utilized with streptococcus MG (3). In all cases definite capsular swelling occurred with S cells in the presence of homologous immune serum but not in the presence of heterologous immune serum. R variant cells showed no evidence of quellung in any of the immune sera employed. As previously noted with streptococcus MG (3), quellung of S cells in homologous serum was more readily seen when the preparations were held for a few hours at 4°C.

TABLE III

Results of Cross-Capsular Swelling Tests with Str. salivarius Type I, Type II, and R Variants Derived from Each

Rabbit serum Immune *versus*	Streptococcal cells	Capsular swelling
Type I (S)	Type I (S)	+++
" " "	" " R var.	0
" " "	Type II (S)	0
Type II (S)	Type I (S)	0
" " "	Type II (S)	+++
" " "	" " R var.	0

Cross-Precipitation Reactions with Capsular Polysaccharides of Type I and Type II.—Capsular polysaccharides were extracted and purified from broth cultures of each type of S cell according to the procedure described above. It should be emphasized that sucrose was not present in the culture medium. The preparations were tested for their capacity to give precipitation with immune sera by the capillary precipitin technique. The mean results of numerous experiments are given in Table IV. Precipitation occurred in all cases only in the presence of homologous immune serum. With type I capsular polysaccharide, a dilution of 10^{-5} regularly gave precipitates with type I (S) serum. Type II preparations seldom gave precipitates at a dilution greater than 10^{-4}.

The results of these various serological reactions indicate clearly that both type I and type II *Str. salivarius* elaborate capsular polysaccharides which confer type specific immunological properties upon the bacterial cells. R variants derived from either type of S cell either do not form a similar substance or produce amounts so small as to be non-demonstrable by the procedures employed.

Cross-Agglutination Reactions with S and R Forms from Sucrose Cultures.— On the basis of the findings recorded in the preceding sections, it appeared possible to undertake a systematic analysis of the immunological properties of the levans formed by *Str. salivarius* types I and II as well as their R variants when grown in the presence of sucrose. The questions submitted to experimental tests were: (1) what is the immunological relationship between the levans formed by type I and type II S cells; (2) are non-encapsulated R variants capable of forming levan; and (3) what is the immunological relationship between levans formed by R variants derived from type I and from type II?

The procedure was identical with that used in cross-agglutination reactions with bacteria grown in the absence of sucrose. In the present experiments immune sera prepared as described above with microorganisms grown either in the presence or in the absence of sucrose were tested with suspensions of streptococci which had been grown either in the presence or the absence of sucrose. In each case a number of sera and a number of streptococcal suspensions were employed and, in so far as was feasible, the cross-agglutination experiments were carried out simultaneously.

TABLE IV

Results of Cross-Precipitation Tests with Capsular Polysaccharides Derived from Str. salivarius Type I and Type II

Rabbit serum	Capsular polysaccharide			
Immune *versus*		Dilution*		
		10^{-3}	10^{-4}	10^{-5}
Type I (S)	Type I (S)	4	4	1
" " "	Type II (S)	0	0	0
Type II (S)	Type I (S)	0	0	0
" " "	Type II (S)	3	3	0

* Dilution = grams per cubic centimeter.

The results of a number of cross-agglutination reactions are shown in Table V. In all cases the type of agglutination observed corresponded either to the S or the R variety described above (*cf.* Table I); no intermediate variety of agglutination was shown. To simplify the presentation of results, the geometric mean of all the observed serum titers is given in either the S or the R column in Table V depending on the type of agglutination which was regularly observed with the immune serum and streptococcal suspension under consideration. The reactions of type I and type II and their R variants grown in sucrose with type I, type II, or R variant immune sera prepared against organisms cultured in *broth* were neither quantitatively nor qualitatively different from those observed with the same systems when all the organisms had been grown

TABLE V

Results of Cross-Agglutination Tests with Str. salivarius Type I, Type II, and R Variants Derived from Each, Grown in Presence or Absence of Sucrose

Rabbit serum	Streptococcal suspension		Geometric mean serum dilution end-point	
Immune *versus*		Grown in broth with added	S type agglutination	R type agglutination
Type I (S)	Type I (S)	Sucrose*	1280	—
" " "	" " R var.	"	—	2560
" " "	Type II (S)	"	50	—
" " "	" " R var.	"	—	40
Sucrose* type I (S)	Type I (S)	None	1280	—
" " " "	" " R var.	"	—	1280
" " " "	Type II (S)	"	10	—
" " " "	" " R var.	"	—	40
Sucrose type I (S)	Type I (S)	Sucrose	1950	—
" " " "	" " R var.	"	1280	—
" " " "	Type II (S)	"	1280	—
" " " "	" " R var.	"	512	—
Type II (S)	Type I (S)	Sucrose	14	—
" " "	" " R var.	"	—	32
" " "	Type II (S)	"	2040	—
" " "	" " R var.	"	—	812
Sucrose type II (S)	Type I (S)	None	25	—
" " " "	" " R var.	"	—	408
" " " "	Type II (S)	"	1550	—
" " " "	" " R var.	"	—	1620
Sucrose type II (S)	Type I (S)	Sucrose	80	—
" " " "	" " R var.	"	2040	—
" " " "	Type II (S)	"	1280	—
" " " "	" " R var.	"	1280	—
R var. type I	Type I (S)	Sucrose	145	—
" " " "	" " R var.	"	—	2560
" " " "	Type II (S)	"	28	—
" " " "	" " R var.	"	—	28
Sucrose R var. type I	Type I (S)	None	128	—
" " " " "	" " R var.	"	—	1820
" " " " "	Type II (S)	"	10	—
" " " " "	" " R var.	"	—	40
Sucrose R var. type I	Type I (S)	Sucrose	280	—
" " " " "	" " R var.	"	1550	—
" " " " "	Type II (S)	"	2040	—
" " " " "	" " R var.	"	1620	—

* Streptococci used for preparation of suspensions were grown in presence of 5 per cent sucrose.

in the absence of sucrose (*cf.* Table I). Similarly, the reactions obtained with immune sera prepared against organisms grown in the presence of sucrose and suspensions made from streptococci grown in *broth* were closely similar in kind and degree to those observed with the same systems when all the bacteria were grown in the absence of sucrose. However, when sera prepared against sucrose-grown streptococci were tested against sucrose-grown streptococci, a markedly different reaction pattern emerged. In all cases the reactions were of the S type and none of the sera distinguished clearly and regularly between S and R variant cells or between S cells of the two types. Under these conditions almost all evidence of an immunological distinction between the two types disappeared as was most clearly apparent with the R variant suspensions and corresponding antisera.

The antigenic relationships between types I and II as well as their R variants and the effect of growth in the presence of sucrose upon the relationships is shown graphically in Fig. 1.

The agglutination titers employed for computation of the r ratios shown are those presented in Tables I and V. The basis for the use of the r ratio and the means by which it is computed are given in detail in an earlier paper (21). This function expresses in a single figure the extent of the antigenic relationship between two strains; the larger the reciprocal of the ratio, $1/r$, the smaller is the relationship.

Cross-Precipitation Reactions with Capsular Polysaccharides and Levans from S and R Forms.—Capsular polysaccharides derived from type I and type II and, in addition, levans synthesized by enzymes obtained from type I and type II as well as their R variants were tested for their capacity to yield precipitates in the presence or in the absence of sucrose.

The technique was identical with that used above in cross-precipitation reactions. A number of sera against each microorganism and a number of preparations of capsular polysaccharides or levans were employed. In certain experiments all of the variables in these cross-precipitation experiments were tested simultaneously.

The mean results obtained in a number of experiments are shown in Table VI. The reactions between capsular polysaccharides obtained from type I and type II were as sharply type specific with immune sera prepared against sucrose-grown streptococci as with similar sera prepared against streptococci grown in broth (*cf.* Table IV). As was anticipated from the agglutination reactions with anti-R variant sera (*cf.* Tables I and V), a small but definite amount of anti-S was present in such sera. This was sufficient to give distinct precipitation reactions with the homologous capsular polysaccharide. The reactions obtained with levans were strikingly different from those observed with the capsular polysaccharides. Irrespective of the source of the enzyme used for the production of levan, there was no indication that the substance possessed type specific properties. Each of the levan preparations reacted in a closely

similar manner with immune sera against sucrose-grown type I or type II organisms as well as the R variant derived from type I; none reacted with any immune serum against streptococci grown in broth.

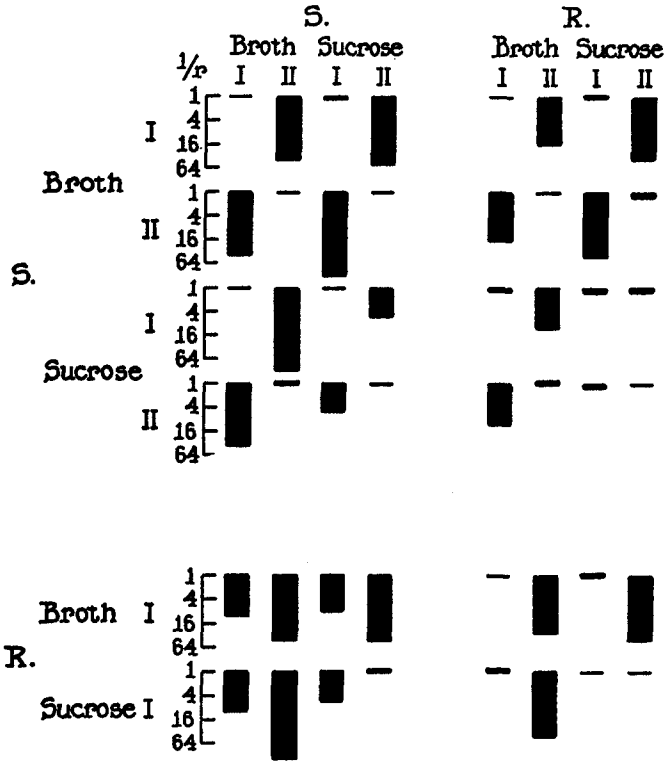

FIG. 1. Graphic representation of the degree of antigenic relationship between type I and type II *Str. salivarius* grown in the presence or absence of sucrose as determined by the titer ratios found in cross-agglutination experiments. S = encapsulated cell; R = non-encapsulated variant; I = type I; II = type II; broth = Todd-Hewitt broth; sucrose = Todd-Hewitt, broth plus 5 per cent sucrose; $1/r$ = recripocal of geometric mean, r, of the two titer ratios, r_1 and r_2, obtained by dividing the heterologous titer by the homologous titer; $r = \sqrt{r_1 \times r_2}$ (21). The length of a column is proportional to the extent of the antigenic dissimilarity between two strains.

The results of absorption experiments confirmed these findings. A typical experiment is recorded in Table VII. Absorption with type I (S) cells removed antibodies capable of reacting with the homologous capsular polysaccharide but did not affect reactions with levans synthesized by enzymes derived from the homologous or heterologous type.

Cross-Complement Fixation Reactions with Capsular Polysaccharides and Levans.—To obtain additional evidence of the immunological dissimilarities

between the capsular polysaccharide and levan derived from the same type of *Str. salivarius*, cross-complement fixation experiments were conducted.

TABLE VI

Results of Cross-Precipitation Tests with Capsular Polysaccharides and Enzymatically Produced Levans Derived from Str. salivarius Type I, Type II, and R Variants

Rabbit serum	Streptococcus	Capsular polysaccharide					Levan (enzymatic)				
Immune *versus*		Dilution					Dilution				
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Type I (S)	Type I (S)	4	4	4	1	0	0	0	0	0	0
" " "	" " R var.						0	0	0	0	0
" " "	Type II (S)	0	0	0	0	0	0	0	0	0	0
" " "	" " R var.						0	0	0	0	0
Sucrose type I (S)	Type I (S)	4	4	4	1	0	4	4	4	3	1
" " " "	" " R var.						4	4	4	3	0
" " " "	Type II (S)	0	0	0	0	0	4	4	4	3	0
" " " "	" " R var.						4	4	4	3	1
R var. (type I)	Type I (S)	3	2	1	0	0	0	0	0	0	0
" " "	Type II (S)						0	0	0	0	0
Sucrose R var. (type I)	Type I (S)	3	2	±	0	0	4	4	4	4	2
" " " " "	" " R var.						4	4	4	3	0
" " " " "	Type II (S)	0	0	0	0	0	4	4	4	4	1
" " " " "	" " R var.						4	4	4	4	1
Type II (S)	Type I (S)	0	0	0	0	0	0	0	0	0	0
" " "	" " R var.						0	0	0	0	0
" " "	Type II (S)	4	3	3	0	0	0	0	0	0	0
" " "	" " R var.						0	0	0	0	0
Sucrose type II (S)	Type I (S)	0	0	0	0	0	4	4	4	4	1
" " " "	" " R var.						4	4	4	3	0
" " " "	Type II (S)	4	4	3	1	0	4	4	4	4	1
" " " "	" " R var.						4	4	4	3	1

The technique was identical with that employed in earlier studies (3). The various polysaccharide preparations were the same as those used in the cross-precipitation experiments described above.

The mean results of numerous experiments are shown in Table VIII. Capsular polysaccharide obtained from either type I or type II gave complete fixation of complement at a dilution of 10^{-6} with homologous immune serum but did not react with immune serum against the heterologous type. In the presence

of immune sera prepared against sucrose-grown streptococci, levans synthesized with enzymes derived from types I or II fixed complement at a dilution

TABLE VII

Results of Precipitation Tests with Absorbed Serum and Capsular Polysaccharides as Well as Levans Produced Enzymatically from Str. salivarius Type I and Type II

Rabbit serum		Streptococcus	Capsular polysaccharide					Levan (enzymatic)				
Immune *versus*	Absorbed with		Dilution					Dilution				
			10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Sucrose R var. (type I)	0	Type I (S)	3	2	1	0	0	4	4	4	4	2
" " " " "	0	Type II (S)	0	0	0	0	0	4	4	4	3	1
" " " " "	Type I (S)	Type I (S)	0	0	0	0	0	4	4	4	4	2
" " " " "	" " "	Type II (S)						4	4	4	4	1
Normal	0	Type I (S)	0	0	0			0	0	0		
"	0	Type II (S)	0	0	0			0	0	0		

TABLE VIII

Results of Cross-Complement Fixation Tests with Capsular Polysaccharides and Levans Produced Enzymatically from Str. salivarius Type I and Type II

Rabbit serum	Streptococcus	Capsular polysaccharide					Levan (enzymatic)					
Immune *versus*		Dilution					Dilution					
		10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Type I (S)	Type I (S)	4	4	4	4	0	4	4	0	0		
" " "	Type II (S)						0	0	0	0		
Type II (S)	Type II (S)	4	4	4	4	0	4	4	0	0		
Sucrose type I (S)	Type II (S)	0	0	0			4	4	4	4	4	0
Sucrose type II (S)	Type I (S)	0	0	0			4	4	4	4	4	0
" " " "	Type II (S)						4	4	4	4	4	0
Normal	Type I (S)	0	0	0			0	0	0			
"	Type II (S)	0	0	0			0	0	0			

of 10^{-7} in both homologous and heterologous immune sera. The slight reactions obtained with levan preparations in the presence of antisera against streptococci grown in broth, which did not in any case exceed a dilution of 10^{-4}, are undoubtedly attributable to the presence of capsular polysaccharide

in the filtrates used as enzyme source (*cf.* Table II). It would be expected that the purification procedure employed would concentrate capsular polysaccharide present in such filtrates along with the levan that was formed from sucrose.

Non-Antigenicity of Purified Levan.—Rabbits were injected intravenously with from 2.5 to 10 mg. of levan synthesized from sucrose with enzyme derived from either type I or type II *Str. salivarius*. In no case did any of the animals develop antibodies capable of precipitating with levan or of agglutinating sucrose-grown streptococci of homologous type. It appears, therefore, that purified levan is non-antigenic in the rabbit under the conditions tested.

Cross-Serological Reactions with Various Types of Non-Hemolytic Streptococci.—The evidence obtained in this investigation and in earlier studies (3, 4) indicates that certain non-hemolytic streptococci possess capsular polysaccharides which are important antigenic constituents and account for the type specific serological reactions shown by these microorganisms. To discover how frequently other non-hemolytic streptococci recovered from the respiratory tract of patients with acute respiratory infections possess an analogous antigenic composition, a number of strains were studied with the serological procedures employed in preceding sections.

Non-hemolytic streptococci were isolated from sputa or throat washings obtained from patients with various acute respiratory infections. The cultural techniques employed were identical with those used previously (2, 6) for the isolation of streptococcus MG from patients with primary atypical pneumonia. Non-hemolytic streptococci were identified on the basis of the following criteria: capacity to grow in a semiselective medium containing gentian violet, sodium azide, and sulfapyridine (6); cultural characteristics on rabbit blood agar plates; morphological and staining properties; growth in chains in liquid media; absence of lysis on prolonged incubation; insolubility in ox bile. All strains were cultured on 5 per cent sucrose agar plates; only those which showed no tendency to produce levan on long incubation were utilized. All streptococcal suspensions were prepared from *broth* cultures containing no sucrose and all immune sera were obtained from rabbits immunized with similar suspensions.

A summary of results of cross-agglutination reactions with 26 strains of non-levan-forming streptococci is shown in Table IX. For comparative purposes, the results of cross-agglutination reactions obtained with types I and II *Str. salivarius* also are shown. Four distinct serological types of non-hemolytic streptococci, exclusive of *Str. salivarius*, were revealed by these experiments. In every instance the agglutination reactions were of the S type, closely similar to those obtained with streptococcus MG (3) or with *Str. salivarius* type I (4) and type II. The almost complete absence of cross-reactions between members of the non-levan-forming group is noteworthy. The slight cross-reactions between types I and II *Str. salivarius* were discussed above and the immunological relation between streptococcus MG and type I *Str. salivarius* has been shown to be attributable to antigenic similarities in their capsular poly-

saccharides (3, 4). Among a total of 33 non-levan-forming strains tested, all but 7 (21 per cent) were readily identifiable by the agglutination technique and fell into one or another of the 4 serological types illustrated in Table IX.

Cross-quellung tests as well as cross-precipitation tests with culture filtrates were carried out with 4 of the MG strains and 2 of the PY strains. In each instance positive type specific reactions were obtained with homologous immune serum as not with heterologous immune serum. These findings are consistent with what would be expected if the large majority of the strains of non-hemolytic streptococci under study elaborate immunologically active and type specific capsular substances. As yet, no attempt has been made to separate and

TABLE IX

Results of Cross-Agglutination Tests with Various Types of Non-Hemolytic Streptococci Obtained from the Respiratory Tract of Man

Rabbit serum	Streptococcal suspension					
	MG	PY	GE	VA	*Str. salivarius*	
Immune *versus*	No. of strains*				Type I	Type II
	18	4	2	2		
MG	**2560**‡	0	0	0	320	0
PY	0	**1280**	0	0	0	0
GE	0	20	**1280**	0	120	0
VA	0	0	0	**320**	0	0
Str. salivarius type I	160	0	60	0	**1280**	20
" " " II	0	0	20	0	10	**1280**

* No strain in this group was capable of forming levan from sucrose.

‡ Reciprocal of serum dilution end-point. 0 = no agglutination at a serum dilution of 1:10.

purify the capsular polysaccharide from any of the 4 serological types other than streptococcus MG (3).

Representative strains of each of the 4 types of non-levan-forming streptococci were tested, by the agglutination technique, against acute phase and convalescent sera from 7 patients with primary atypical pneumonia. Each of the patients selected had developed a significant antibody response against streptococcus MG (6). In none of the sera were antibodies demonstrable against any type other than MG.

It would be of interest to determine whether any of the serological types illustrated in Table IX correspond with the serological *groups* reported by Solowey (11). As yet, there has been no opportunity to carry out the necessary cross-experiments. Because the acid extraction technique of Lancefield (22) extracts but does not destroy the capsular polysaccharide of streptococcus MG

(3), it appears possible that the serological *groups* delineated by Solowey (11) with this technique are in reality serological types and may also be attributable to immunologically different capsular polysaccharides.

DISCUSSION

That the antigenic constitution of certain non-hemolytic streptococci, including *Str. salivarius*, is analogous to that of encapsulated pneumococci appears clear from the results of this and earlier studies (3, 4). At least six serological types of these bacteria can be identified positively and differentiated clearly from other non-hemolytic streptococci by means of any one of a number of classical immunological procedures. In addition, antibodies directed against each species can be distinguished and their concentration measured with considerable reproducibility by relatively simple *in vitro* techniques.

Str. salivarius type I and type II (1) each possess the capacity to produce two serologically distinct and apparently unrelated polysaccharides which appear to be disposed at or near the surface of the bacterial cell. One, the capsular polysaccharide, in close relationship with the bacterial cell, develops along with the bacterium as growth progresses in ordinary nutrient media, and endows the microorganism with its type specific immunological properties. The other, a levan, develops only when the bacterium is grown in the presence of sucrose (or raffinose) (13) and, although highly reactive in serological tests with appropriate immune sera (14), seems to have no place in the antigenic structure of the bacterium when sucrose is absent from the medium.

The levan develops when an enzyme elaborated by and released from the cell acts on an appropriate substrate (sucrose) (14). So far as can be determined, the levan is immunologically unrelated to the capsular polysaccharide of type specific streptococci which produce it. Moreover, the levan is produced in the presence of sucrose by R variants which are either incapable of forming capsular polysaccharide or produce only minimal amounts. In the case of *Str. salivarius* (either S or R forms), the enzyme, levanase, is elaborated by the bacterial cell and released into the culture medium even when sucrose is absent. With certain other species of bacteria this is not the case and the enzyme appears only when the appropriate substrate is present (19). What function this highly active polysaccharide-synthesizing enzyme may play in the economy of the bacterial cell in the absence of sucrose is as yet unknown. Enzymatically synthesized levan is not antigenic on intravenous injection in the rabbit although that present at the bacterial surface after growth in sucrose is highly antigenic under similar experimental conditions. This situation is analogous to that which prevails with capsular polysaccharides derived from type specific pneumococci or streptococcus MG (3). Like levan, these substances are non-antigenic in the rabbit when separated from the bacterial cell but are highly immunogenic when present at the cell surface.

Despite the almost complete lack of any demonstrable immunological relationship between the capsular polysaccharides of type I and type II *Str. salivarius*, as well as the dissimilarities of the antigens present in their non-encapsulated R variants, there appears to be no immunological difference between the levans formed by the two types or their R variants. These results are in accord with the findings of other workers (14–16) which indicate that levans formed by a wide variety of bacterial species possess similar serological properties.

SUMMARY

The type specific immunological properties of certain non-hemolytic streptococci, including *Str. salivarius* type I and type II, present in the respiratory tract of human beings appear to be dependent upon the presence of capsular polysaccharides.

The levans formed from sucrose by *Str. salivarius* (encapsulated S cells or non-encapsulated R variants), or by cell-free enzymes derived from these microorganisms, are indistinguishable immunologically and show no evidence of type specificity. Such levans appear to be immunologically distinct from and unrelated to the capsular polysaccharides of the microorganisms which produce them.

BIBLIOGRAPHY

1. Sherman, J. M., Niven, C. F., Jr., and Smiley, K. L., *J. Bact.*, 1943, **45,** 249.
2. Mirick, G. S., Thomas, L., Curnen, E. C., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1944, **80,** 391.
3. Mirick, G. S., Thomas, L., Curnen, E. C., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1944, **80,** 407.
4. Mirick, G. S., Thomas, L., Curnen, E. C., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1944, **80,** 391.
5. Curnen, E. C., Mirick, G. S., Ziegler, J. E., Jr., Thomas, L., and Horsfall, F. L., Jr., *J. Clin. Inv.*, 1945, **24,** 209.
6. Thomas, L., Mirick, G. S., Curnen, E. C., Ziegler, J. E., Jr., and Horsfall, F. L., Jr., *J. Clin. Inv.*, 1945, **24,** 227.
7. Loewe, L., Plummer, N., Niven, C. F., Jr., and Sherman, J. M., *J. Am. Med. Assn.*, 1946, **130,** 257.
8. Hehre, E. J., and Neill, J. M., *J. Exp. Med.*, 1946, **83** 147.
9. Birkhaug, K. E., *J. Infect. Dis.*, 1927, **40,** 549.
10. Hitchcock, C. H., *J. Exp. Med.*, 1928, **48,** 393.
11. Solowey, M., *J. Exp. Med.*, 1942, **76,** 109.
12. Lancefield, R. C., *J. Exp. Med.*, 1933, **57,** 571.
13. Niven, C. F., Jr., Smiley, K. L., and Sherman, J. M., *J. Bact.*, 1941, **41,** 479.
14. Hehre, E. J., *Proc. Soc. Exp. Biol. and Med.*, 1945, **58,** 219.
15. Hehre, E. J., Genghof, D. S., and Neill, J. M., *J. Immunol.*, 1945, **51,** 5.

16. Genghof, D. S., Hehre, E. J., and Neill, J. M., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61,** 339.
17. Sevag, M. G., Lackman, D. B., and Smolens, J., *J. Biol. Chem.*, 1938, **124,** 425.
18. Niven, C. F., Jr., Smiley, K. L., and Sherman, J. M., *J. Biol. Chem.*, 1941, **140,** 105.
19. Hestrin, S., Avineri-Shapiro, S., and Aschner, M., *Biochem. J.*, 1943, **37,** 450.
20. Swift, H. F., Wilson, A. T., and Lancefield, R. C., *J. Exp. Med.*, 1943, **78,** 127.
21. Archetti, I., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1950, **92,** 441.
22. Lancefield, R. C., *J. Exp. Med.*, 1928, **47,** 469.