

THE IMMUNOLOGICAL RELATIONSHIPS OF STREPTOCOCCUS VIRIDANS AND CERTAIN OF ITS CHEMICAL FRACTIONS.

II. SEROLOGICAL REACTIONS OBTAINED WITH ANTINUCLEOPROTEIN SERA.

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In the preceding paper (1) a study of the reactive and antigenic constituents of *Streptococcus viridans* was made by an analysis of the antibody content of antibacterial sera. It was shown that such sera contain relatively non-specific as well as specific antibodies. The specific antibody, apparently a single one, is probably concerned with the parallel phenomena of agglutination and precipitation of the serum by the specific carbohydrate chemically extracted from the cell. The non-specific antibody shows group reactions with nucleoproteins of serologically distinct strains.

In order to investigate the group relationships of the nucleoproteins, it seemed advisable to immunize rabbits to this fraction of the bacterial cell. The preparation of this material has already been described (1). The method of immunization was to give repeated series of daily intravenous injections of small doses for 4 or 5 days, followed by a rest of 3 days. Usually four to six series were required to produce antisera of suitable titer. These sera deteriorate on standing in the ice box for a few months. Table I shows the results obtained in titrating four such sera against protein antigens. The chief points of interest were as follows:

1. Cross-precipitation was universal. The homologous antigen was usually slightly more effective than heterologous ones, but the difference was not marked and was probably due to the peculiarities of individual antigens resulting from unavoidable denaturation in

TABLE I.
Precipitins in Sera of Rabbits Immunized with P.

Antigen dilution.	Rabbit L59. Immunized with P from Strain W67.					Rabbit L63. Immunized with P from Strain A148.				
	Antigen for precipitin test. P from Strain					Antigen for precipitin test. P from Strain				
	W67	A148	A49	38D	<i>S.</i> <i>hæmo-</i> <i>lyticus.</i>	W67	A148	A49	38D	<i>S.</i> <i>hæmo-</i> <i>lyticus.</i>
1:200	0	0	0	0	0	—	—?	—?	±	—
1:500	0	0	0	0	0	—	—?	—?	—	—
1:1,000	0	0	0	0	0	—?	±	±	—	—
1:2,000	0	0	0	0	0	±	+	+	—	+?
1:5,000	++++	+++	+++?	+?	++±	±	0	±	±	+?
1:10,000	+++	++±	+++?	+?	++	±	±	+	—	+?
1:20,000	++±	+	++	+?	+	±	+	±	—	—
1:40,000	±	±	±	—	±	—	±	—?	—	—
1:80,000	—	—	—	—	—	—	—	—	—	—

Antigen dilution.	Rabbit L57. Immunized with P from Strain A49.					Rabbit L65. Immunized with P from Strain 38D.				
	Antigen for precipitin test. P from Strain					Antigen for precipitin test. P from Strain				
	W67	A148	A49	38D	<i>S.</i> <i>hæmo-</i> <i>lyticus.</i>	W67	A148	A49	38D	<i>S.</i> <i>hæmo-</i> <i>lyticus.</i>
1:200	0	0	0	0	0	—	—	—	+?	—
1:500	0	0	0	0	0	—	—	—	+	±
1:1,000	0	0	0	0	0	±	±?	±	++	+
1:2,000	0	0	0	0	0	+	±	+	+++±	++
1:5,000	++	+++	+++	±	++	++	+?	+±	+++±	+++±
1:10,000	++	++	++±	+?	+±	+±	+?	+	+±	+±
1:20,000	+	+	++	—	+?	+	±	+?	±	+
1:40,000	±	±	—?	—	±	—?	—	±	—	+

All strains in this table except *Streptococcus hemolyticus* are *Streptococcus viridans*.

The precipitations were performed by mixing 0.2 cc. of antigen dilution with 0.1 cc. of serum and 0.1 cc. of physiological salt solution. All dilutions were made with physiological salt solution. The tests were incubated for 2 hours at 37°C. and read after standing 18 hours in the ice box. In all experiments complete sets of controls with and without normal serum were included. Unless the controls were negative, the experiment was not considered as evidence. The controls have been omitted from the tables in order to save space.

All dilutions are recorded as final dilutions.

The experiments recorded in this table and Table V were performed in the following way. Serum was diluted 1:3 and antigen added to make a final concentration of 1:4,000. The precipitin titrations had shown that these were the optimal dilutions for precipitation. The tubes were incubated for 2 hours at 37°C. and then placed in the ice box overnight. After removal of the precipitate by centrifugation, antigen was again added to a final concentration of 1:4,000. The tubes were again incubated for 2 hours at 37°C. and left overnight in the ice box. This procedure was repeated until no further precipitate was formed. Usually three or four absorptions were sufficient to remove all antibodies for the antigen used as absorbent. Precipitin tests were made with 0.3 cc. of the absorbed serum (the equivalent of 0.1 cc. of original undiluted serum) added to 0.1 cc. of suitable antigen dilutions. Several antigens were titrated in final dilutions of 1:2,000, 1:4,000, and 1:8,000 against representative lots of absorbed serum. Only one dilution is recorded for each test since consistent results were obtained in all tubes. Readings were made after 2 hours at 37°C. and overnight in the ice box.

preparation. Thus antigen P from Strain 38D was suspected of having undergone considerable denaturization; the results in Table I show that it precipitated heterologous sera only slightly, and that its antiserum was only slightly precipitated by other antigens.

2. Prozone phenomena were observed. At an antigen dilution of 1:200 or even 1:1,000 no precipitation occurred in the serum of Rabbits L63 and L65, while at a dilution of 1:5,000 good precipitations were found throughout the series. Similar prozones occurred in all antiprotein sera.

3. In a given serum, the titration curves were almost identical for the various antigens. A protein antigen from hemolytic streptococcus showed the same curves as the non-hemolytic antigens.

Complement fixation tests with the same series of antigens and sera confirmed the findings of Table I. In addition it was found that none of these sera was precipitated by S (the soluble specific substance) from any source.

An absorption experiment was performed with the best sera of this series (Rabbit L59 serum), using as antigens the protein solutions prepared from four different strains of non-hemolytic streptococcus. After six successive treatments of a sample of this serum with its homologous protein solution, no further precipitate was obtained on addition of the homologous protein or of any heterologous green streptococcus protein. Proteins from three different strains were used in similar experiments to absorb samples of the same anti-P serum (Rabbit L59 serum) with the result that each heterologous protein removed all P antibodies from the serum. These absorption experiments confirmed the indications of the precipitin and complement fixation tests that the nucleoproteins of non-hemolytic streptococci are serologically similar.

With the cooperation of Dr. Avery, who furnished pneumococcus protein solutions, proteins from Types I, II, and III pneumococcus and from a Group IV strain were used as antigens in precipitin tests against the four green streptococcus anti-P sera then available. Each protein solution precipitated all four sera, an indication of group relationship between these species. More information was obtained concerning the relationship of pneumococcus and streptococcus proteins by absorption experiments with another antiprotein serum.

Rabbit L90 was immunized with the original unpurified N/100 sodium hydroxide extract of a non-hemolytic streptococcus. Tables II and III show the result of testing this serum by means of precipitin reactions and absorption tests with protein antigens from four non-hemolytic streptococci, from four strains of hemolytic streptococci, from three different staphylococci, and finally from a strain of *Bacillus coli* and from one of *Bacillus diphtheriae* as controls. The antiserum against the green streptococcus protein was also tested with pneumococcus protein. The hemolytic streptococcus strains were from the following sources: one, scarlet fever, N.Y.5, obtained from Dr. A. R. Dochez, one erysipelas, and the other two were representatives of two of the groups studied by Dochez, Avery, and Lancefield in 1919 (2). Another anti-P serum prepared against protein from a hemolytic streptococcus, was tested with all these antigens except the pneumococcus protein. The results are shown in Tables IV and V. From the precipitin tests alone, it seemed that the hemolytic and the non-hemolytic streptococcus proteins were interchangeable; both antisera were precipitated equally well by proteins from either group. Pneumococcus protein precipitated the serum of Rabbit L90 (Table II) better than any other antigen in the series. Protein from staphylococcus, on the contrary, was consistently less reactive than either of the streptococcus antigens, while the proteins from unrelated groups represented by strains of *Bacillus coli* and *Bacillus diphtheriae*, failed to precipitate these sera in any dilution. It is noteworthy that in this series all proteins giving positive results of any sort precipitated the serum to approximately the same dilution of antigen. In spite of the fact that the recorded antigen dilutions are not entirely accurate, since they are based on weights of dried nucleoproteins rather than on chemical determinations of protein nitrogen present in the solutions, the difference in the several antigens seemed to be represented not by the range of antigen dilution over which precipitation occurred but rather by the amount of precipitate produced.

Absorption tests made with each serum and the twelve antigens of the four groups detailed above, gave more exact information concerning the interrelationships than was afforded by precipitin tests. The results are recorded in Tables III and V. By these experiments it was shown that absorption with protein from any organism of the

group used in immunization removed all antibodies from the serum. For example, in absorption tests with the serum of Rabbit P976, immunized against protein from a hemolytic streptococcus, every hemolytic streptococcus protein absorbed all antibodies for hemolytic and non-hemolytic streptococcus proteins and for staphylococcus

TABLE IV.

Precipitin Titration.

P antibodies in serum of Rabbit P976.

Immunized with P from *Streptococcus hemolyticus* S3.

Antigen: P from	Antigen dilution.					
	1:1,000	1:2,000	1:4,000	1:8,000	1:16,000	1:32,000
<i>Streptococcus hemolyticus</i> S3.	+++?	++	++	+++?	+	+
“ “ S60.	++	++	+++	++	+	±
“ “ N. Y. 5.	++	++	+++±	+++±	++	+
“ “ A.	+++	+++	+++±	+++±	++	++
“ <i>viridans</i> A49.	+	±	++	++	++	±
“ “ W67.	±	±	++	±	±	±
“ “ A148.	+	±	++	++	±	+
“ “ 38D.	±	+	++	++	++	±
Staphylococcus R.	+	±	+	+	+	±
“ A.	±	±	±	±	+	+
“ H.	±	±	+	±	±	±
<i>B. coli.</i>	-	-	-	-	-	-
“ <i>diphtheriae.</i>	-	-	-	-	-	-

The technique described for precipitin tests in Table I was used in this experiment.

proteins as well. A green streptococcus protein, however, absorbed all antibodies for green streptococcus and for staphylococcus proteins but only reduced the titer for hemolytic streptococcus protein without completely removing the antibodies for the latter. A much smaller proportion of the total antibody was absorbed by staphylococcus protein; it removed all staphylococcus antibodies and reduced the titer slightly for proteins of the other groups. A diagram (Fig. 1)

TABLE V.
Absorption Experiment.

P antibodies in serum of Rabbit P976.
Immunized with P from *Streptococcus hemolyticus* S3.

Antigen in precipitin test. Dilution 1:4,000. P from Strain	Controls.		P antibodies in serum of Rabbit P976 after absorption with P from															
	Unabsorbed serum of Rabbit P976.	Normal rabbit serum.	<i>Streptococcus hemolyticus</i> Strain				<i>Streptococcus viridans</i> Strain				Staphylococcus Strain							
			S3	S60	N.Y.5.	A	A49	W67	A148	38D	R	A	H					
<i>Streptococcus hemolyticus</i> S3.	++	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
" " S60.	++±	-	-	-	-	-	-	-	-	0	+	+	+	±	+	+	+	+
" " N.Y.5.	+++	-	-	-	-	-	-	-	-	0	+	+	+	+	+	+	+	+
" " A.	+++	-	-	-	-	-	-	-	-	0	+	+	+	+	+	+	+	±
" <i>viridans</i> A49.	++±	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
" " W67.	±±	-	-	-	-	-	-	-	-	0	-	-	-	-	+	+	+	+
" " A148.	+++	-	-	-	-	-	-	-	-	0	-	-	-	-	+	+	+	+
" " 38D.	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	±
Staphylococcus R.	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
" A.	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
" H.	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Saline control.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

The technique described in Table III was used in this experiment.

makes this relationship clearer. Staphylococcus protein contains some fraction, represented in the diagram as A, in common with the two kinds of streptococci. This common fraction must be a small part of the whole complex, as shown by the slight precipitate and by the slight reduction in titer for streptococcus antigens after absorption of the serum with staphylococcus protein. The hemolytic and non-hemolytic streptococci have some rather large fraction B in common as indicated by heavy cross-precipitation and the large amount of cross-absorption. However, a certain residue remains which is distinctive for each kind of nucleoprotein. The distinctive parts of the

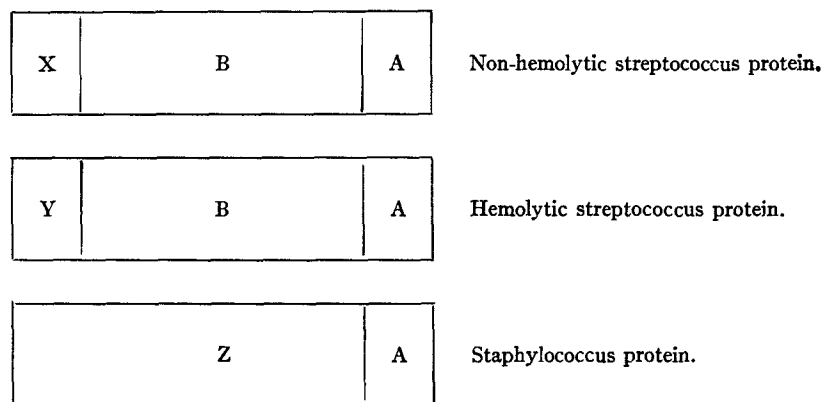


FIG. 1. Diagram to illustrate results of absorption experiments with anti-protein sera. No attempt has been made to diagram the relationship of pneumococcus protein to the proteins represented above since insufficient data are available for this purpose.

proteins are represented diagrammatically as X, Y, and Z for proteins from the different organisms. The structure of the diagram is probably the same for all members of a given group indicating that the proteins of any one group are serologically similar. No diagram has been made to show the relationship of pneumococcus protein to proteins from the other Gram-positive cocci, since insufficient data are available for more than a general statement of its immunological relationship. Tables II and III show that pneumococcus protein has fractions in common with the other proteins studied, in that cross-precipitation and cross-absorption were observed in the experiment with the serum from Rabbit L90. Pneumococcus protein seemed

more closely related to green streptococcus protein than to the protein of any other group and in addition the pneumococcus and streptococcus proteins showed closer relationship, one with the other, than was found between the proteins of any other groups studied.

It has been found impossible to produce an antiserum for staphylococcus protein of sufficient strength to carry out an absorption experiment. The cross-precipitations, using a weak staphylococcus serum against the series of proteins tested previously, gave indications that crossing occurs, but the antibody content of the serum was too slight to permit satisfactory absorption tests.

From these indications of the group relationships of the proteins of the Gram-positive cocci, it seems reasonable to suppose that the extensive group crossing among the hemolytic and the non-hemolytic streptococci demonstrated by Kinsella and Swift (3, 4) is referable to the delicacy of the complement fixation test in detecting protein antigens and antibodies. Hitchcock's (5) demonstration, with the same test, of group relationships between the streptococci and the pneumococci is explainable on the same basis as due to the presence of similar protein fractions in these closely related species.

Non-Antigenic Nature of S.

The antinucleoprotein sera furnished indirect evidence concerning the production of antibodies by injection of S. The nucleoprotein antigens used in the preparation of antisera all contained demonstrable quantities of S, not completely removed in the course of purification. The six rabbits immunized with nucleoprotein received between twenty-five and thirty intravenous injections over periods of 6 to 8 weeks. No S antibodies appeared in their sera. In order to determine whether S can function as an antigen after dissolution of the cell complex, Rabbit L90 was immunized with the original unpurified N/100 sodium hydroxide extract of pulverized bacteria. At no time during immunization could S antibodies be demonstrated in the serum of this animal, although the material injected contained in solution all the S obtainable by this method from the bacterial sediment of 7 liters of original broth culture. This is at least indirect evidence that S from non-hemolytic streptococci does not, after dissociation from the cell, lead to antibody production. On account of the limited

supply of purified material no direct experiments have been performed yet in the attempt to immunize with chemically isolated S. However, the evidence from the antiprotein sera gives the same indications as the results of others who have tried to immunize with S. Avery, (6) after repeated and extensive efforts, has reported complete lack of success in immunizing with this material from pneumococcus; Zinsser and his associates (7-10), failed to produce antibodies to their bacterial "residue antigens;" Mueller and Tomcsik (11) did not succeed in immunizing rabbits with their purified specific carbohydrate from yeast; in fact, no one has thus far been able to produce S antibodies except by injection of the intact cell body.

DISCUSSION.

The significance of the results obtained with both antibacterial and antinucleoprotein sera are considered in this discussion and summary.

A study of the different kinds of antigens and their corresponding antibodies has shown an antigenic complex for *Streptococcus viridans* entirely analogous to that found in pneumococcus by Avery and Heidelberger (12, 13). Certain differences occur, due perhaps in some measure to differences in "cell architecture" (6), but in its main outlines the situation is the same. Under appropriate conditions, two different antibodies may be produced: one an antibody for the bacterial protein which shows group reactions with proteins from related species, and the other an antibody for the non-protein, probably carbohydrate substance. This soluble specific substance is only antigenic when in the unaltered cell complex. The antibody for the latter is precipitated by the specific soluble substance chemically extracted from the cell and is closely related to specific agglutination of the homologous bacteria. Specific agglutination and S precipitation are parallel. Table VI summarizes the antibody response to the two kinds of antigens. Living bacteria stimulate in the rabbit specific agglutinins and S antibodies, which are probably identical according to absorption experiments; but intact bacteria may also lead to the production of non-specific antibodies whose presence can be demonstrated by precipitation and complement fixation with the protein fractions of homologous and heterologous strains. It is possible that P antibodies in such a serum may be responsible for slight non-specific cross-agglutination.

The study of the antigenic complex of the non-hemolytic streptococci has been extended to include the relationships between the proteins of the common pathogenic Gram-positive cocci. The sera prepared for this purpose against cell extracts largely composed of protein (although some S is always present as an impurity) give rise solely to protein antibodies which may be demonstrated either by the precipitin or the complement fixation reaction. Cross-precipitation and absorption experiments with these sera have been used to show certain group relationships among the proteins of related Gram-positive cocci. That cross-precipitation is not a common characteristic of nucleoproteins from all bacterial species is shown by the lack of cross-reactions with proteins prepared from organisms of unrelated groups. It is not yet known whether the serological relationships of

TABLE VI.
Antigenic Value of Cell Constituents of Streptococcus viridans.

Rabbit.	Serum tested for					
	Agglutinin.		Precipitin.			
	Homologous.	Heterologous.	S		P	
Homologous.			Heterologous.	Homologous.	Heterologous.	
Immunized with						
Living bacteria.	++++	±?	++++	-	++	+
P (containing S as an impurity).	±	-	-	-	++++	++++

similar protein fractions in related species are dependent upon separate chemical compounds or upon certain radicals possessed in common. There is a possibility that the cross-reactions are entirely due to denaturation of the native protein by chemical manipulation. Even if this is true there must be some fundamental similarity in the original proteins which leads to similar products of denaturation. In this connection it should be recalled that certain antibacterial sera with a high content of P antibodies show indiscriminate cross-precipitation within the group but only slight reactions when tested against proteins from related bacterial species. In antibacterial sera, in which the untreated intact bacterium is used in immunization, the response must be reflected in antibodies more nearly directed against the native

bacterial protein than is possible in sera prepared by immunization with the chemically treated protein extracts which are subject to denaturation in the process of preparation. It is hard to interpret the facts relating to cross-relationships among the proteins, since the results obtained by use of the antibacterial sera do not altogether confirm the results shown by sera prepared against the chemically extracted proteins. The majority of the evidence, however, seems to indicate group relationships based on immunological similarities in the bacterial proteins.

Only a small amount of homologous agglutinins have been found in anti-P sera. Potent sera of this kind usually agglutinate the homologous strain in the lower dilutions, but give no agglutination with heterologous strains. The failure of anti-P sera to agglutinate heterologous strains may be correlated with the better precipitation of antibacterial sera by proteins from homologous strains than from heterologous. However, in view of the evidence from cross-precipitation and absorption experiments with both kinds of antisera, one must conclude that although serologically the bacterial proteins of different strains of green streptococci are not necessarily identical, they are nevertheless closely related.

The structure of the cell probably plays an important rôle in determining the amount of agglutination that may be caused by P antibodies. Thus it has been found (6) that in ordinary pneumococcus cultures no real anti-P agglutination occurs, but rather a precipitation caused by the ready autolysis of pneumococcus suspensions and the consequent liberation of protein into solution. However, pneumococci which by adventitious circumstances have been deprived of all S-producing function and simultaneously of the surrounding capsule, are agglutinable by P antibodies. In streptococcus, the distribution of S material is probably different from that in pneumococcus. In the latter, the S substance is chiefly present in the capsule while the streptococcus has no such ectoplasmic zone. Attempts to obtain the S substance from streptococci indicate that this material is distributed through the cell body, or at least is in the interior, since rupture of the cell itself is necessary in order to obtain the S substance in any quantity. With such a distribution of cellular materials, it is conceivable that in the streptococcus some of the protein constituents are

at the surface of the cell and render it, therefore, subject to the action of P antibodies and subsequent agglutination.

No S antibodies have been found in anti-P sera, although in one instance the whole cell extract was used for immunization without any removal of S. No direct attempts to produce S antibodies have been made for streptococcus, but the results with the anti-P sera are indicative of the probable futility of such attempts with the purified material, especially in view of the failure of all other workers to produce antibodies with purified specific substances.

Certain attempts have been made to determine the chemical nature of the specific soluble substance. Only qualitative tests could be made with the limited supply available, but the preliminary chemical findings are submitted. This substance is not only serologically entirely analogous in its behavior to the pneumococcus soluble specific substance, but chemical examination has shown that it is non-protein by the ordinary tests and that it contains carbohydrates which give reducing sugars on hydrolysis. Such a substance is strikingly suggestive of the polysaccharides described by Heidelberger and Avery (13, 14). All the evidence so far accumulated indicates the possibility of a similar chemical composition for the soluble substance of the non-hemolytic streptococcus.

SUMMARY.

1. The immunological behavior of two cell constituents of non-hemolytic streptococci has been studied. (a) One, the so called nucleoprotein, is relatively non-specific and gives rise to an antibody which shows group reactions with nucleoproteins of related species. (b) The other is non-protein by qualitative chemical tests. Preliminary chemical examination has indicated that it may be a carbohydrate. Although this substance is highly reactive with the specific antibodies produced by the intact bacterial cell, yet in its chemically purified condition it is probably non-antigenic. Specific serological reactions with this substance are closely related to specific agglutination of the microorganism.

2. The study of sera prepared by immunization with the chemically extracted protein has shown the presence of antibodies for nucleoproteins alone. No antibodies against the specific soluble substance

have been found in these sera. The protein antibodies are little, if at all, concerned in causing agglutination. Precipitin tests, complement fixation reactions, and absorption experiments have been used to analyze the group relationships with the nucleoproteins of other species. The proteins of each species of Gram-positive cocci studied were immunologically similar within the species and showed definite relationships to the proteins from related species. Proteins from bacteria of unrelated species did not react with antisera against streptococcus protein.

3. Two distinct antibodies have been demonstrated in antisera prepared against living bacteria. By prolonged immunization it was found possible to produce sera with a high content of protein as well as specific antibodies. With ordinary methods, however, the immune sera had a low content of relatively non-specific protein antibodies but a high titer for specific antibodies. The specific antibodies were not reactive with proteins but were active with high dilutions of the soluble specific substance and were responsible for the parallel specific agglutination. Absorption experiments showed that the two antibodies in antibacterial sera were immunologically distinct.

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