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

I. SEROLOGICAL REACTIONS OBTAINED WITH ANTIBACTERIAL SERA.

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This analysis of the reactive and antigenic substances obtainable from non-hemolytic streptococci was begun as a result of previous work which showed the presence in the serum of certain rheumatic fever patients of complement binding antibodies for streptococcus extracts. The earlier work, to be reported in detail elsewhere, showed that positive reactions were obtained in a variety of diseases when nucleoprotein extracts of *Streptococcus viridans* cultures were used as antigens. These cross-reactions indicated the desirability of investigating the entire antigenic complex of *Streptococcus viridans* in a manner similar to that employed by Avery and Heidelberger (1–3) in their work on pneumococcus. As a preliminary to a satisfactory evaluation of the importance of certain types of streptococcus antibodies in the serum of rheumatic fever patients, it was necessary to know what antibodies an antistreptococcus serum might contain. This could best be determined by a study, on the one hand, of the reactive and antigenic substances present in cultures and in extracts of non-hemolytic streptococci, and, on the other hand, by a study of the antibodies evoked in rabbits in response to immunization with cultures and their chemical fractions. It seemed desirable also to ascertain whether the conclusions of Avery and Heidelberger in regard to pneumococcus were applicable to another species of bacteria, or whether there would be discovered in a heterogenous group, such as the green streptococci, a set of conditions different from those existing in a species with sharply defined types.

In the work referred to, Avery and Heidelberger (2) found a nucleoprotein fraction common to all types of pneumococci. The sera of animals immunized with purified nucleoprotein from one type gave equally good reactions with protein from any other type. Type specificity, however, was found due to the presence of the soluble specific substance of Dochez and Avery (4) which has recently been subjected to intensive chemical and immunological analysis by Heidelberger and Avery (1, 3). This substance is a complex carbohydrate which is chemically, as well as immunologically, distinctive for each type of pneumococcus, and is serologically active in very high dilutions. In contrast to the antigenic *species-specific* nucleoprotein, this *type-specific* carbohydrate has never been found to incite the production of antibodies after separation from its cell complex.

A number of other investigators have reported highly reactive specific substances present in culture filtrates or in extracts of bacterial sediments. In a few instances such substances have also been found in the blood and urine of patients, as well as in the body fluids of experimental animals. Fornet (5) in 1906 found precipitable substances in the blood and urine of typhoid fever patients and in the blood of experimentally infected rabbits. Ascoli (6), and Hamburger (7) as well, had shown that precipitinogen passed through the kidney and could be demonstrated in the urine. Dochez and Avery (4) in 1917 isolated type-specific substances from young actively growing pneumococcus cultures and from the blood and urine of pneumonia patients and of pneumococcus-infected rabbits.

Specific precipitable substances in culture filtrates have been studied extensively since the early work of Kraus; but recently attention has been particularly focussed on substances obtained from culture filtrates or from extracts of bacterial or other cell bodies as being causally related to specificity. Thus Zinsser (8) and his associates (Parker (9), Mueller (10), Mueller and Tomcsik (11), Petroff (12)) have shown that "residue antigens" from a variety of bacteria, and also from yeast, are non-protein specifically reactive substances which are not antigenic in chemically purified condition, although they incite the production of antibodies while still in the unaltered state within the cell. The work in this field of Dochez and Avery and of Heidelberger and Avery has already been mentioned. Hitchcock (13) also has tested relatively protein-free precipitating substances from a number of non-hemolytic streptococci and has found an array of heterogenous strains by this method. He has also studied (14) substances from hemolytic streptococci which are apparently chemically similar to "residue antigens" from other bacteria but which differ serologically in that cross-reactions are obtained with extracts from immunologically distinct groups of hemolytic streptococci. The meningococci have been analyzed from the same point of view by Przesmycki (15) with similar results: a practically type-specific substance has been found for each group. Landsteiner, van der Scheer, and Witt (16) have demonstrated recently that alcoholic extracts of human red blood cells contain the specific reactive substances, probably lipoidal in nature, for the blood groups of man. Thus the substances responsible for specificity have been isolated in varying degrees of chemical purity from a number of bacterial species, from yeast cells, and from red blood cells.

A systematic immunological study of the less specific nucleoprotein fraction has been made only for pneumococcus. Avery and Heidelberger (2) have demonstrated that the chemically extracted nucleoproteins of pneumococcus show *species specificity* rather than *type specificity*. Furthermore, this fact has been related to certain instances of non-specific agglutination (17) in that pneumococci which have lost their capsules and the function to produce the specific substance show non-specific agglutination due to the uncovering of the protein contained in the bacterial bodies. Other authors have reported group reactions which are probably in a similar manner directly dependent on the presence of related proteins. Kinsella and Swift (18, 19) by means of the complement fixation reaction showed certain group relationships among the non-hemolytic streptococci and among the hemolytic streptococci as well. Hitchcock (20), using the same method, has recently reported complicated group relationships between the hemolytic and the non-hemolytic streptococci and the pneumococci. Meyer and Christiansen (21) on the basis of their study of cutaneous hypersensitiveness of rabbits to typhoidin and similar preparations believe that all bacteria must contain some protein substances in common. Eberson (22), after studying the effect of ultraviolet rays on the antigenic properties of meningococci, concluded that a single protein structure represents the element common to groups of biologically related organisms.

This review of the literature indicated that many bacteria contain a highly reactive specific substance which can be extracted chemically and that at least one species, pneumococcus, contains another fraction common to the entire species. Scattered observations were also found which tended to show, less conclusively than for pneumococcus, that the protein fractions of some other species were responsible for group reactions. It seemed important therefore to determine the basis of group and specific reactions of the non-hemolytic streptococci both from the point of view of their possible connection with rheumatic fever and from a more general consideration of the immunological relationships involved.

Methods.

Four strains of *Streptococcus viridans* were selected. Of these, two (A49 and 38D) were isolated by Dr. H. F. Swift from blood cultures of rheumatic fever patients and two were obtained from blood cultures in cases of bacterial endocarditis. All were methemoglobin producers, were bile-insoluble and had the fermentation properties recorded in Table I. Various other strains were used incidentally, but these four were most carefully studied.

Rabbits were immunized with from three to six doses of living broth cultures

injected intravenously twice a week. The first dose was 3 to 5 cc. with subsequent doses usually double the preceding one until a total of 20 cc. was reached; by this time the animals were usually highly immune; they were bled from 5 to 12 days after the last injection.

The materials were extracted from the bacteria according to Woolridge's (23) method for the nucleoproteins and Heidelberger and Avery's (1, 3) method for the soluble specific substance, with certain modifications required for this kind of organism. From 3 to 12 liters of an 18 hour plain broth culture were centrifuged, the bacterial sediment put into a flask with about 2 cc. of sterile sand, and dried *in vacuo* at a temperature not exceeding 37°C. This dry mass was then ground in a ball mill to a fine powder in from 1 to 7 days. Then 100 cc. of N/100 sodium hydroxide were added for every 3 liters of original culture and the flask agitated overnight in the cold. The suspension was centrifuged at high speed, and the sediment of sand and bacterial detritus discarded. The opalescent supernatant

TABLE I.

Fermentation Reactions of the Four Strains Chiefly Studied.

Strain.	Milk.	Raffinose.	Inulin.	Salicin.	Mannitol.	Lactose.	Saccharose.
W67	+	+	−	+	−	+	+
A148	+	+	−	−	−	+	+
A49	+	−	−	−	−	+	+
38D	+	−	−	+	−	+	+

+ indicates acid and clot; −, neither acid nor clot.

Hiss serum water containing 1 per cent of the test substance was used for testing the fermentation properties of these strains. The tubes were incubated at 37°C. for 7 days. Daily observations were made during the period of incubation.

fluid was treated in the cold with the minimal amount of 10 per cent acetic acid necessary to cause complete precipitation; a final concentration of about 0.5 per cent acetic acid was usually required. The precipitate, the so called nucleoprotein fraction, consisting of a mixture of all the bacterial substances precipitated by acetic acid in the cold, was further purified by washing with distilled water or physiological salt solution, redissolving in dilute sodium hydrate solution and reprecipitating with acetic acid. This process was repeated once or twice, the final precipitate washed in water, then very rapidly in acetone and dry ether, after which it was dried *in vacuo*. The resulting fine white powder was stored as the stock material in a vacuum desiccator and subsequently made into solutions by weight as desired. An average yield from 9 liters of original culture was 400 to 500 mg. A few lots were diluted and filtered through Berkefeld V filters at some stage in the preparation, but since considerable loss was experienced by this method and the filtered product gave the same serological results as the unfiltered,

this procedure was abandoned. This antigen has been designated as the nucleoprotein fraction, or for brevity as P. According to Wells (24), such a solution is probably composed of nucleoproteins, nucleins, mucins, "nucleoalbumins," various glycoproteins besides the mucins, simple globulins, and alkaline proteinate formed by the action of alkali on the native protein.

The soluble specific substance was purified from the crude supernatant fluid left after precipitation of the nucleoprotein. With the streptococci it was necessary to recover this substance from the bacterial bodies rather than from the culture fluid, since only a small amount of it is found in solution in streptococcus cultures. The material derived from 18 liters of original broth culture was combined in one lot. The crude solution was evaporated on a steam bath to a small volume (about 50 cc.) and acidified with acetic acid in the cold; and any precipitate was discarded. The clear solution was then boiled in a water bath for 10 minutes in order to remove heat-coagulable proteins, which were also discarded. The supernatant fluid was treated with 10 volumes of 95 per cent alcohol; the resulting precipitate was redissolved in 5 to 10 cc. of water and any insoluble matter discarded after washing with water. The solution, which stood overnight in the ice box between precipitations, was again acidified with dilute acetic acid and again treated with alcohol. This process was repeated about six times, with half the precipitations on the acid and half on the alkaline side. The watery solution was then dialyzed in a parchment bag against running water for 48 hours, or until tests for chlorides, phosphates, and sulfates were negative. In some cases the solution was filtered through a Berkefeld V filter at this stage and concentrated *in vacuo* to about 10 cc.; then one-fourth its volume of 1:1 hydrochloric acid was added in an attempt to precipitate the specific substance, since the Type III pneumococcus specific substance precipitates with this treatment. However, none of the lots so treated with hydrochloric acid showed any precipitate. After standing in the ice box overnight, the hydrochloric acid was removed by dialysis against running water, the solution concentrated to 5 cc. or less, and treated with 10 volumes of acetone. The precipitate was taken up in 1 or 2 cc. of distilled water, reprecipitated with acetone, and dried in a vacuum desiccator to constant weight. The yield from 18 liters of original broth culture was 25 to 30 mg. of flaky yellowish white material. This substance dissolved in physiological salt solution was water-clear and colorless. The solution (referred to for convenience as S) was tested serologically and, to a limited extent, chemically. The presence of further impurities was indicated by a slight residue, insoluble in water, which still remained after the last precipitation.

Easier methods of obtaining the soluble specific substance were sought. In spite of the fact that streptococcus cultures do not autolyze, as pneumococcus cultures do, it was thought that enough S might be secreted by the organism to make the culture filtrate a profitable source of the material. Flasks of broth, inoculated respectively with strains W67, A148, A49, and 38D, were incubated at 37°C.; at intervals of 2 or 3 days during the succeeding month, samples were withdrawn and tested with an antibacterial serum for the presence of a reacting

substance; but only small amounts could be demonstrated at any time. Rapid freezing and thawing of the sedimented bacteria suspended in a small volume of saline gave almost no reacting substance in the supernatant fluid after centrifugation, even though the suspension was frozen and thawed as may as twenty times. Boiling a concentrated suspension of streptococci with various reagents caused the dissociation of S from the bacterial cell. Merely boiling for an hour with physiological saline was effective; although the addition of sodium hydrate or sodium carbonate gave a greater yield. Dissolving the bacteria in antiformin and precipitating out the proteins was also tried successfully. In short, any method of breaking up the cell body is effective in liberating the soluble specific substance. In extraction by boiling with sodium hydrate, the soluble specific substance of certain strains was found to be destroyed, or at least inactivated to a great extent. Consequently, it was felt inadvisable to subject the material to such strong chemical agents as boiling sodium hydroxide or antiformin, and the more tedious method of grinding the dried bacteria and subsequently extracting with N/100 sodium hydroxide in the cold was adhered to throughout.

Solutions of the specific substance prepared as described were subjected to as many qualitative chemical tests as possible with such a small amount of material. In a dilution of 1:400, preparations from the four strains gave negative biuret reactions and positive Molisch tests for carbohydrates. The latter was positive to approximately the limit of serological reactivity of the substance. A positive Molisch test was obtained at a dilution of 1:400,000 with a solution which gave a precipitin test no higher than 1:500,000. Weakly positive reactions for reducing sugars were obtained from Strains W67 and 38D; but after hydrolysis by boiling with concentrated acid strongly positive reactions were obtained with Fehling's solution from all four strains. No precipitate was obtained with phosphotungstic acid in any instance, but with copper sulfate and uranium nitrate a precipitate was formed in the solutions made from Strains W67 and A148 but not with those from A49 and 38D. These solutions gave no color with iodine and were not precipitated with 1:1 hydrochloric acid. The specific substance passed through a Berkefeld V filter and withstood autoclaving and boiling for prolonged periods. Preparations from some strains withstood boiling with N/10 sodium hydroxide for an hour. In dialysis experiments the specific substance was held back by ordinary parchment membranes.

This necessarily meager chemical information at least gives the same indications as the studies of Heidelberger and Avery on pneumo-

coccus. It seems probable that the specific substance of non-hemolytic streptococcus is non-protein in nature. That it may be a polysaccharide is indicated by the positive Molisch test in high dilution together with the presence of reducing sugars after hydrolysis. The differences in precipitation of preparations from different strains by heavy metal salts is interesting in view of similar distinctions between the specific substances from Type II and Type III pneumococci. More detailed chemical analysis must await the collection of large quantities of material.

The serological behavior of the bacterial substances, separated and prepared as noted in the preceding section, was studied by means of sera from rabbits immunized, on the one hand, against the living bacteria and, on the other, against the chemically extracted protein material. Preliminary tests had indicated that the two kinds of antibodies found by Avery and Heidelberger in antipneumococcus sera were also present in sera obtained by immunizing rabbits with living cultures of green streptococci: one antibody for the highly reactive strictly specific soluble substance (S), the other for the less highly reactive relatively non-specific nucleoprotein (P). The antibacterial sera against the four organisms chiefly studied were now tested for antibody content. Twelve antibacterial sera were titrated completely for agglutinins, for S precipitins, and for P precipitins. A number of other rabbit sera, used incidentally, were not titrated completely; but in so far as these were used the results were consistent with those from the sera more thoroughly studied. Two typical protocols of complete titrations are shown (Tables II and V). The records of the other sera are omitted because in their essential features they do not differ from those shown. The one exception will be considered in some detail.

Table II shows the results for the serum of Rabbit L71, immunized with living bacteria of Strain W67. Tested for agglutinins against the four strains in question, it showed a high titer with the homologous organism, at least 1:4,000 serum dilution, and no appreciable cross-agglutination with the other three strains. Similarly precipitation tests with the purified specific substance showed absolute specificity in relatively high dilutions. In this particular serum a good precipitation was obtained only as far as 1:100,000 dilution of S; but

in many sera a titer of 1:500,000 has been obtained, and in a few instances there was undoubted precipitation at 1:1,000,000. This is

TABLE II.

Antibody Content of Antibacterial Serum.

Rabbit L71, immunized for a short period with living culture of Strain W67.

Agglutinin.					Precipitin.									
Serum dilution.	Culture from Strain				Antigen dilution.	S from Strain				Antigen dilution.	P from Strain			
	W67	A148	A49	38D		W67	A148	A49	38D		W67	A148	A49	38D
1:20	++++	–	–	+	1:5,000	++++	–	–	–	1:1,000	+++	±	–	–
1:40	++++	–	–	+	1:10,000	++++	–	–	–	1:2,000	++	±	+	–
1:80	+++	–	–	+	1:50,000	++	–	–	–	1:4,000	++	–	–	–
1:160	+++	–	–	+	1:100,000	+	0	0	0	1:8,000	++	–	–	–
1:1,000	+++	0	0	0	1:500,000	–	0	0	0	1:16,000	+	–	–	–
1:4,000	++	0	0	0						1:32,000	±	–	–	–
1:16,000	+	0	0	0										
1:32,000	±	0	0	0										

++++ indicates complete agglutination or precipitation.

+++, ++, +, ± indicates grades in the series to –, which indicates a negative reaction.

0 indicates that no test was made.

S = soluble specific substance. P = nucleoprotein.

In this table, as in all subsequent ones, the agglutinations were performed by mixing 0.5 cc. of serum dilution (made with broth) with 0.5 cc. of an 18 hour plain broth culture. The tubes were incubated for 2 hours at 56°C. and read immediately.

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.

low in comparison with the 1:6,000,000 titers of pneumococcus S observed by Avery and Heidelberger (17), but their figures were obtained with more highly purified material and with serum of horses

subjected to long periods of immunization. In working with rabbits immunized much less intensively and with antigens of no higher degree of purification than those used in this study, it is felt that these figures are comparable to their results.

Much lower titers for P than for S antibodies were obtained in antibacterial sera. The titration of Rabbit L71 serum illustrates this point. Here again significant precipitation was found only with the homologous nucleoprotein, although there was a trace of cross-precipitation with proteins from Strains A148 and A49. The same marked difference has been observed in the character of the precipitate obtained from precipitation of the serum by S and by P as that described by Avery (17) for pneumococcus precipitates. The S precipitate is typically a compact, slightly translucent disk at the bottom of the tube. The flakes which form immediately, after mixture of S and serum settle out very quickly and coalesce to a considerable extent by the end of the 2 hour incubation period. After the tubes have stood overnight in the ice box, it is almost impossible to break up this mass by shaking the tube. This is the condition when precipitation is complete. The incomplete precipitates show varying degrees of compactness and also different amounts of precipitate. This point has been discussed by Morgan (25) for pneumococcus; and Hitchcock (13, 14) has reported the same kind of precipitate in his experience with streptococcus. In contrast to this compact disk, the P precipitate is composed of heavy flocculi which do not coalesce but form a bulky mass of finely divided particles easily shaken up from the bottom of the tube. This difference between S and P precipitates is striking and constant.

The lack of cross-precipitation with nucleoprotein antigens in an antibacterial serum, such as that of Rabbit L71, was a distinct surprise since earlier work using the complement fixation reaction and similarly prepared antigens and sera had clearly indicated consistent cross-fixation of antibacterial sera by heterologous P antigens. Ten rabbits were used in the complement fixation series, with two or three immunized against each organism. Of these, five gave strongly positive cross-fixations, while the other five showed moderate or weak, but still very definite cross-reactions. A protocol of the complement fixations with the serum of one of these rabbits giving strong reactions

is shown in Table III. The proper proportion of antigen and serum for precipitin tests had not been determined at the time this serum was available; but the precipitations indicated only slight reactions with the method used. This is perhaps not surprising in view of the fact that the complement fixation reaction is a more delicate test than precipitin tests.

From the result of the complement fixation reactions it seemed probable that the serum of Rabbit L71 would give some cross-precipitation with P from heterologous strains of *Streptococcus viridans;* but since little cross-precipitation was actually observed, the possibility was suggested that the precipitation with the homologous P antigen was due to traces of S remaining in the nucleoprotein solution and that the serum did not contain any antibodies for P. Accordingly, an attempt was made to remove all traces of S remaining as an impurity in the nucleoprotein antigen derived from W67. The protein was precipitated with acetic acid and carried through a number of additional purifications by washing with saline, redissolving, reprecipitating, and finally dissolving the purified nucleoprotein in its initial volume. All wash waters and supernatant fluids after the various precipitations were tested for S in order to determine the amount of S present as an impurity in the original neucleoprotein solution, as well as to be more certain that the final purified solution did not contain demonstrable quantities of S. The three wash waters were neutralized and tested against serum from Rabbit L71. The original supernatant fluid from the first acetic acid precipitate was not tested in this way. Table IV shows a heavy precipitate with the first wash water, and only slight precipitates with the two succeeding ones. But the wash waters of a nucleoprotein precipitate usually contain varying amounts of P in solution. Consequently, it was thought that probably some of this precipitate was due to P, while some might be due to S. In order to determine how much was due to S, the original supernatant fluid and the wash waters were treated with acetic acid in the cold and then boiled for 10 minutes, still in the acid condition, to remove proteins. After neutralization, the solutions were again tested against the serum of Rabbit L71. Only slight precipitates now appeared. This was interpreted as an indication that most of the precipitate with the first untreated wash water was not attributable

TABLE III.

Complement Fixation Reactions.

Rabbit 49, immunized for a short period with living culture of Strain A148.

Serum.	Antigen.			
	P from Strain			
	W67	A148	A49	38D
cc.				
0.1	+++	++++	++++	++++
0.05	++++	++++	++++	++++
0.025	+++	++++	++++	+++
0.012	+++	++++	++++	+++
0.006	++	++++	+++	+
0.003	++	+++	++	−

In the complement fixation experiments, ¼ Wassermann quantities were used. 0.25 cc. of a 1:500 dilution of antigen was used in all tubes. Antigen, guinea pig complement, and immune serum were mixed in a total volume of 0.75 cc. and incubated in a water bath at 37°C. for 1 hour. 5 per cent washed sheep cells were sensitized by mixing with an equal volume of an appropriate dilution of rabbit anti-sheep serum. 0.5 cc. of sensitized cells was added to all tubes of the test, followed by a secondary incubation period of ½ hour. Readings were made after standing overnight in the ice box.

A complete set of controls was included using normal serum, and antigen and serum alone in at least double the largest amounts used in the test. All controls were satisfactory.

TABLE IV.

P from Strain W67 Tested for Traces of S.

Precipitin tests.

	Untreated wash waters.			Wash waters treated with acetic acid cold and boiling.				Purified P.
	1	2	3	Original supernatant fluid.	1	2	3	
Serum of Rabbit L71........	+++	±	+	−?	±	+	−	++±

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

to S but to P. The final purified P, presumably free of S, gave as good a precipitate with Rabbit L71 serum as the unpurified control. Nucleoprotein antigens from the other strains gave similar results when tested in the same way against their respective antisera. It seemed apparent, therefore, that the P preparations contained only negligible amounts of S as an impurity, insufficient to account for the specific reactions sometimes obtained with nucleoprotein antigens. Whatever precipitate was present when P was used as antigen in the precipitin test against its homologous antibacterial serum was then chiefly a P precipitate. One must conclude, therefore, that homol-

TABLE V.

Antibody Content of Antibacterial Serum.

Rabbit L92, immunized for a long period with living culture of Strain A148.

Agglutinin.					Precipitin.									
Serum dilution.	Culture from Strain				Antigen dilution.	S from Strain				Antigen dilution.	P from Strain			
	W67	A148	A49	38D		W65	A148	A49	38D		W67	A148	A49	38D
1:40	+	+++	++	−	1:5,000	−	++++	−	−	1:500	+	+++	++	+
1:80	+	+++	++	−	1:10,000	−	++++	−	−	1:1,000	+±	++++	++	+
1:160	−	+++	++	−	1:50,000	−	++++	−	−	1:2,000	+	+++	+	+
1:320	−	+++	++	−	1:100,000	−	++	−	−	1:4,000	+	++	+	+
1:640	−	++	+	−	1:500,000	0	+	0	0	1:8,000	+	++	+	±
1:1,280	−	++	±	−						1:16,000	+	+	+	−
1:2,560	−	±	−	−										

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

ogous nucleoproteins react better than heterologous ones with an antibacterial serum. The reason for this is not entirely clear but this fact seems to indicate individual differences in proteins from different strains, especially in the light of other evidence to be discussed later.

It was, however, possible to obtain sera which showed marked cross-precipitation with heterologous nucleoproteins. The serum of Rabbit L92, immunized over a long period against Strain A148, is an illustration of this point (Table V). Several similar sera have been prepared.

An instance of cross-agglutination was found which corroborated the existing information on the parallelism between agglutination and S precipitation. Immunization with a green streptococcus (V92) isolated from an excised subcutaneous nodule of a rheumatic fever patient in 1923 gave rise to a serum which was precipitated by the soluble specific substance of one of the main strains studied (38D). Five sera against V92 were prepared. Reciprocal cross-precipitation of S was demonstrated; cross-agglutination tests gave the same result. It is evident, therefore, that strains of non-hemolytic streptococci which cross-precipitate the soluble specific substance also cross-agglutinate. The converse had already been shown.

The only exception to these general results of antibody content in the antibacterial sera was encountered in the serum of Rabbit L95, an animal highly immunized by a prolonged course of treatment with Strain A49. As may be seen in Table VI, this serum showed cross-precipitation with S from Strain A148. The corresponding cross-agglutination was slight with the usual technique; but when the incubation period was doubled, or the readings delayed for 18 hours following 2 hours incubation at 56°C., cross-agglutination was more pronounced than with the ordinary method. In Table VI both readings are given.

The anti-P content of this serum should be specially noted. Marked precipitation occurred with P from heterologous strains (Table VI). In fact, better precipitation was observed with P from Strain A148 than with P from the homologous strain. This cross-precipitation of proteins with an antibacterial serum should not be overlooked in evaluating the protein group relationships indicated by study of pure anti-P sera. In antibacterial sera the P antibodies are produced in response to the native protein of the unaltered bacterial cell. Evidence derived from the study of these antibodies should, therefore, be given special weight. Table VI shows that proteins from related Gram-positive cocci react only slightly with this serum, although the same series of antigens gives striking cross-reactions when tested with sera prepared against the chemically extracted proteins.

A careful study of this serum by means of absorption tests with bacteria both from Strain A49 and from Strain A148 failed to throw light on the peculiarities of this exceptional serum. Consequently,

TABLE VI.

Antibody Content of Antibacterial Serum.

Rabbit L95, immunized for a long period with living culture of Strain A49.

Agglutinin.							Precipitin.				
Serum dilution.	Read after 2 hrs. at 56°C.				Read after 2 hrs. at 56°C. and 18 hrs. in the ice box.		Antigen dilution.	S from Strain			
	Culture from Strain				Culture from Strain						
	W67	A148	A49	38D	A148	A49		W67	A148	A49	38D
1:40	–	±	++++	+++	+++	++++	1:5,000	–	++	++++	–
1:80	–	+	++++	±	+++	++++	1:10,000	–	++	++++	–
1:160	–	±	++++	±	++±	++++	1:50,000	–	+	+++	–
1:320	–	+	++++	–	++	++++	1:100,000	0	+	+++	–
1:640	–	±	+++	–	+±	+++	1:500,000	0	+	+	0
1:1,280	–	–	+++	–	+	+++	1:1,000,000	0	0	±	0
1:2,560	–	–	++	–	±	++	1:2,000,000	0	0	–	0
1:5,120	–	–	+	–	0	0					
1:10,240	–	–	±	–	0	0					

Precipitin for P antigen.

Antigen dilution.	*Streptococcus viridans.*				*Streptococcus hæmolyticus.*				Staphylococcus.			Pneumococcus.	
	P from Strain				P from Strain				P from Strain			Antigen. dilution.	Group IV, Pn. IV.
	W67	A148	A49	38D	S3	S60	N.Y.5.	A	R	A	H		
1:133	0	0	0	0	+	+	+	+	++	++	+	1:5,600	+±
1:200	0	0	0	0	0	±	+	±	++	++	+	1:11,200	+
1:500	++	+++	+++	++	±	±	+	–	++	+	+	1:22,400	±
1:1,000	++	++++	++++	++	–	–	–	–	+	–	±	1:44,800	±
1:2,000	++	++++	++++	+	–	–	–	–	±	–	–	1:89,600	±
1:4,000	+	+++	++	+	0	0	0	0	0	0	0		
1:8,000	+	++	++	+	0	0	0	0	0	0	0		
1:16,000	+	++	+	±	0	0	0	0	0	0	0		

The technique described in Table II for precipitin tests was used in this experiment, except in reading part of the agglutination tests as noted in the table.

it is believed that this is probably an example of a single animal giving an unusual response to immunization. Table VII shows the result of an absorption experiment performed with the serum of this rabbit

(L95). Complete removal of agglutinins by repeated absorptions with the homologous organism (A49) resulted in the removal from the serum of all antibodies including those for the heterologous S from Strain A148 which cross-precipitated with this serum. Absorption

TABLE VII.

Absorption Experiment.

Rabbit L95, immunized for a long period with living culture of Strain A49.

Lot.	Rabbit L95 serum.	Agglutination.							Precipitation.		
		Tested against Culture A49.							Antigen dilution.		
									Strain A49.		Strain A148.
		Serum dilution.							S	P	S
	Absorbed with	1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:10,000	1:1,000	1:12,500
1	Bacteria, Strain A49 (homologous organism).	–	–	–	–	–	–	–	–	–	–
2	Bacteria, Strain A148 (heterologous organism).	+++	++	++	++	+	+	+	++?	–	–
3	Untreated controls Rabbit L95 serum.	++++	++	++	++	+	+	+	++	++	+
4	Normal rabbit serum.	–	–	–	–	–	–	–	–	–	–

This experiment was performed by absorbing the serum in a dilution of 1:4 with the bacterial sediment from the strain indicated for 2 hour periods at 37°C. Nine repetitions were required in order to remove all agglutinins from Lot 1. All other lots were given the same amount of heating and dilution. The sediment from 500 cc. lots of plain broth culture, heated 1 hour at 56°C., was used for the first seven absorptions. The sediment from 3 liter lots was used for the last two absorptions.

under the same conditions with bacteria from the heterologous strain did not affect the agglutinins nor the S precipitins for the strain used in immunization but removed the S antibodies for Strain A148 and all P antibodies as well. The latter fact tends to confirm the belief that the nucleoproteins of the non-hemolytic streptococci are essen-

tially alike in their serological behavior. The absorption of S antibodies for Strain A148 by bacteria from Strain A49 cannot be considered indicative of subgroup relationships since the sera of none of the four other rabbits immunized with Strain A49 showed any cross-precipitation with S from Strain A148. As stated above, it is felt that this rabbit must be one which has given an unusual response to immunization, due to the peculiarities of the individual animal.

An attempt was made to correlate the serological relationship between S and P antibodies and agglutinins in antibacterial sera by means of absorption experiments. An experiment was performed with the serum of Rabbit L71, immunized with living bacteria of Strain W67. Table VIII, Part 1, shows the results after several absorptions with S and P. After four absorptions the lot treated with the homologous S failed to give any further S precipitate, but still precipitated with P as strongly as the control. This serum still gave good agglutination of the homologous strain. It is uncertain whether this agglutination was due to the P antibodies, demonstrable in the serum at that time, or to traces of S antibody no longer detectable by the precipitin test. Lot 2 of the serum after complete absorption of P antibodies showed undiminished reactivity with S, and agglutination of about the same strength as the untreated control. Table VIII, Part 2, gives the final result of this experiment. Lot 1 after complete absorption with S was further treated with P to remove the P antibodies from the serum. Similarly, Lot 2 after complete removal of P antibodies was treated with S to remove the S antibodies, a procedure which removed the agglutinins in both cases.

This experiment brought out several important points:

1. Absorption with S did not remove P antibodies.
2. Absorption with P did not remove S antibodies.
3. The S antibody was closely related to the production of agglutination.

Detailed daily tests in this and in other similar experiments showed that small traces of S antibody are probably sufficient to produce almost maximal agglutination, while P antibodies are little, if at all, effective in causing agglutination. These facts are construed to mean that the antibodies responsible for S precipitation are probably also operative in agglutination.

TABLE VIII.

Absorption Experiment.

Rabbit L71, immunized for a short period with living culture of Strain W67.

Lot.	Rabbit L71 serum.	Agglutination.					Precipitation.	
		Tested against Culture W67.					Antigen dilutions.	
		Serum dilution.					S	P
	Absorbed with homologous	1:40	1:80	1:160	1:320	1:640	1:5,000	1:400
	Part 1. Incomplete absorption.							
1	S	++	++	++	++	+	−	++±
2	P	++	++	+++	++++	++++	++++	−
3	Untreated controls. Rabbit L71 serum.	++	+++	++++	++++	++++	++++	++±
4	Normal rabbit serum.	−	−	−	−	−	−	−
	Part 2. Complete absorption.							
							S	P
							1:5,000	1:200
1	S to completion, then P.	±	±	−	−	−	−	−
2	P to completion, then S.	+	+	+	±	−	−	−
3	Untreated controls. Rabbit L71 serum.	++++	++++	++++	++++	++++	++++	++±
4	Normal rabbit serum.	−	−	−	−	−	−	−

Serum was diluted 1:2 and appropriate amounts of antigen added to make the final antigen concentration the optimal for precipitation. The tubes were incubated for 2 hours at 37°C. and left in the ice box overnight. After the precipitate had been removed by centrifugation, more antigen was added and the procedure repeated until no further precipitate formed. The sera were then tested for agglutinins and for S and P precipitins as indicated in Part 1 of the table. The remaining serum in Lots 1 and 2 was further absorbed with P or with S, as indicated in Part 2 of the table, until absorption was complete. These sera were again tested for agglutinins and for S and P precipitins as recorded in Part 2 of the table.

In conducting the absorption experiments, it was apparent from the first that the procedure was difficult and had many possibilities of error. While not many such experiments have been performed, it is felt, nevertheless, that they are of value in that they confirm the data obtained by other serological reactions.

SUMMARY.

1. Agglutination and precipitation by the specific substance of *Streptococcus viridans* are parallel phenomena. Separate specific substances have been extracted from strains which are distinct by ordinary serological tests. Preliminary chemical examination indicates that the specific substances may be complex carbohydrates.

2. A close relationship between nucleoproteins from different strains of *Streptococcus viridans* is suggested by the existence of a certain amount of cross-precipitation and a larger degree of cross-complement fixation. But the occurrence of stronger reactions with homologous nucleoproteins than with heterologous indicates that there is some degree of individual difference in proteins from separate strains.

3. Two distinct antibodies are present in the sera antibacterial for *Streptococcus viridans:* one of high titer implicated in the parallel phenomena of agglutination and precipitation by the soluble specific substance, the other usually of low titer and involved in precipitation by nucleoproteins but probably little, if at all, in agglutination.

The significance of these results obtained from the study of antibacterial sera will be considered in the general discussion of the antigenic components of *Streptococcus viridans* after the results from the study of antinucleoprotein sera have been presented in the succeeding paper.

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