THE ANTIGENIC COMPLEX OF STREPTOCOCCUS HÆMOLYTICUS.

I. Demonstration of a Type-Specific Substance in Extracts of Streptococcus hæmolyticus.

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Chemical and immunological studies to determine the nature of the substances responsible for the specific reactions of a number of microorganisms have been reported recently by several investigators. Most of this work has been reviewed in some detail elsewhere (1). In certain very carefully studied instances, such as pneumococcus (2) and the Friedländer bacillus (3), specificity is determined by type-specific carbohydrates, while non-specific group reactions are due to species-specific proteins. The same general relationship holds for the *Streptococcus viridans* (4).

Hitchcock (5) found that nearly all hemolytic streptococci yielded in crude antiformin extracts a "residue antigen," such as described by Zinsser and Parker (6), which reacted with all of his antibacterial sera prepared against hemolytic streptococci but not with sera against green streptococci. Conversely (7) similarly prepared "residue antigens" from non-hemolytic streptococci did not react with his hemolytic streptococcus antisera.

Hirsch (8) also reported finding a precipitating substance in extracts of hemolytic streptococci which he called the soluble specific substance. He did not give any evidence to support this statement other than the fact that it precipitated antibacterial sera and sera prepared against solutions of this material. He did not state whether his sera and antigens were all prepared and tested with one or with several strains.

In repeating the work of Hitchcock as a preliminary to the present experiments, it was found, indeed, that all partially purified antiformin extracts, or extracts prepared in such a way as to remove the bulk of the protein, precipitated equally well antibacterial sera made against

different types of hemolytic streptococci. The precipitates formed typical discs like those obtained with type-specific carbohydrates in other species of bacteria. All attempts to separate a type-specific fraction from such extracts by fractional alcoholic precipitations were unsuccessful. Methyl and ethyl alcoholic extracts of whole bacteria contained the same species-specific substance but no trace of a type-specific fraction.

It was then found that extracts made by Porges' method (9) for the removal of bacterial capsules contained a type-specific fraction. This report is the presentation of some of the work carried out with this fraction.

The strains used were chiefly those which had been previously grouped by agglutination and protection tests (10). These had been preserved for 4 or 5 years by dessicating them while in the frozen state by Swift's (11) technique. Cultures recovered from this stock, as a rule, retained the virulence and cultural and serological behavior characteristic of them at the time of dessication. These old strains were used in preference to freshly isolated ones because the distinctive types had already been separated and grouped. The results obtained by any new method of grouping must, of necessity, agree with those obtained by previous methods in order to establish its validity.

Methods.

Antigens were prepared from the various strains by a modification of Porges' method as follows: bacteria centrifuged from 18 hour plain broth cultures, were suspended in 0.85 per cent NaCl solution to which sufficient N/1 HCl was added to make a final concentration of N/20 HCl. Usually the sediment from 1 liter of broth culture was extracted in 10 cc. to 15 cc. volume. The suspension in a Pyrex centrifuge tube was immersed in boiling water for 15 minutes with occasional stirring, then cooled, centrifuged, and the supernatant fluid removed and neutralized with N/1 NaOH. The precipitate which appeared on neutralization was thrown down in the centrifuge; and the water-clear, slightly yellowish supernatant fluid was used as antigen for precipitin tests. When a more potent antigen was desired, it was obtained by extracting in smaller volume or by concentrating the dilute extract described above. For concentration, crystals of sodium acetate—usually 10 gm. per liter of extract—were added to facilitate the subsequent precipitation with three or four volumes of 95 per cent alcohol. After standing overnight in the ice box, the alcoholic precipitate was thrown down and redissolved in salt solution. The greater part of the alcoholic precipitate was insoluble; but

precipitin tests showed that very little loss of active material was occasioned by discarding the insoluble part. The alcoholic precipitation was repeated and the

TABLE I.

Precipitin Reactions with Unabsorbed Antibacterial Serum from Rabbit Q867, Immunized with Strain S60, Type S60.

Antigen: HCl extract			Read after 2 hrs. at 37°C.	Read after additional 18 hrs. in ice box
Strain	Type	Cc.		
S60	S60	0.4	+++	+++
		0.3	+++	+++
		0.1	++	++
S43	"	0.4	++	+++
		0.3	+++	+++±
		0.1	++	++
S23	S23	0.4	−	±
		0.3	−	+
		0.1	±	++
S65	"	0.4	−	+
		0.3	−	+±
		0.1	±	++
S3	S3	0.4	−	±
		0.3	−	+
		0.1	±	++
S144	"	0.4	−	±
		0.3	−	+
		0.1	±	++
S24	Unclassified	0.4	−	±
		0.3	−	+±
		0.1	±	++
S276	"	0.4	−	±
		0.3	−	+
		0.1	±	++

In all tables ±, +, ++, +++, ++++, indicate degrees of reaction; − indicates a negative reaction; 0 indicates that the test was not made.

antigen concentrated to any desired volume by this means. In the following experiments, most of the antigens were the original extracts, although a few were concentrated by the alcoholic precipitation method.

Antibacterial sera were prepared by inoculating rabbits intravenously with increasing doses of heat-killed broth cultures followed by living organisms. After 6 or 8 weeks of immunization, the sera of such animals contained agglutinins and precipitins and, in some instances, protective antibodies for the homologous strain. Precipitins for antigens from strains of unlike type were also present in most of these sera. Experiment 1 gives the typical precipitin reactions of the kind of antibacterial serum which gave only moderate cross-reactions with heterologous antigens.

TABLE II.

Precipitin Reactions with Unabsorbed Antibacterial Serum from Rabbit Q609, Immunized with Strain S23, Type S23.

Antigen: HCl extract			Read after 2 hrs. at 37°C.	Read after additional 18 hrs. in ice box
Strain	Type	Cc.		
S23	S23	0.4	++++	++++
		0.1	+++	+++
S65	"	0.4	+++	+++
		0.1	++	++
S60	S60	0.4	+	++
		0.1	+±	++
S128	"	0.4	+±	++
		0.1	++	++±
S3	S3	0.4	−	±
		0.1	+	++
S144	"	0.4	−	±
		0.1	+	++
S24	Unclassified	0.4	++±	++±
		0.1	+++	+++
S276	"	0.4	±	++
		0.1	+	+±

Experiment 1.—Eight HCl extracts were made from two strains of each of three types of hemolytic streptococcus and from two unclassified strains. Three dilutions of each antigen were set up as follows: 0.4 cc., 0.3 cc., and 0.1 cc. were placed in successive tubes, and the volumes made up to 0.4 cc. with salt solution. 0.1 cc. of serum from Rabbit Q867, immunized as indicated in Table I, was added to each tube; and, after mixing, the tubes were incubated in a 37°C. water bath for 2 hours. The tests were read immediately, and again after standing overnight in the ice box. Controls of serum alone, of antigens alone, and of antigens with normal serum were negative.

Table I shows that antigens of the type homologous to the serum formed heavy flocculent precipitates almost as soon as they were mixed with the serum. The maximum reaction with these antigens was reached during the initial incubation at 37°C., and the intensity changed very little during the ensuing period in the ice box. Heterologous antigens, on the contrary, precipitated the serum slowly, often forming no visible precipitate during the 2 hour period in the water bath, but, after standing overnight in the ice box, they gave disc precipitates similar to those formed by specific carbohydrates of some species of bacteria. The optimum zone for disc precipitates was usually in considerably higher dilutions than for the more nearly type-specific flocculent precipitates.

Experiment 2.—A similar experiment was performed with a slightly different series of antigens and antibacterial serum from another rabbit, immunized as shown in Table II. The same series of tests was set up as in Experiment 1, except that the tube containing 0.3 cc. of antigen was omitted.

The reactions shown in Table II were typical of another kind of antibacterial serum with which immediate pronounced cross-reactions were obtained. Considerably less specificity was evident in the precipitin reactions in this instance than in Experiment 1, in which the 2 hour reading seemed quite type-specific; but, in Experiment 2, this reading showed a large amount of cross-reaction. On the basis of this test the classification of Strains S60 and S128 was doubtful; and Strain S24 would have been placed in *Type* S23 if other evidence had not been available. No conclusion could be drawn from the character of the precipitate in these instances, since it had much the same appearance for the heterologous antigens as for the homologous. Some other means of distinction was necessary.

Accordingly, isolation of the type-specific antigen was attempted but was only partly successful. Fractional alcoholic precipitations served to separate the non-type-specific disc-forming substance; but no satisfactory method was devised for removing the substance which gave non-type-specific flocculent precipitates with immune serum; hence attempts were made to prepare type-specific antisera by absorption. Table III shows such an experiment.

Experiment 3.—Serum from Rabbit Q309, immunized as shown in Table III, was absorbed with a heterologous hemolytic streptococcus. The bacteria from 1.5 liters of plain broth culture of a heterologous strain were centrifuged, resuspended in a small volume of salt solution, and killed by heating at 56°C. for 1 hour. To the packed bacteria, 2 cc. of immune serum diluted with 4 cc. of salt solution were added. A parallel absorption was performed at the same time with bacteria from the strain homologous to the serum. Controls of immune serum and of normal serum similarly diluted were included, and the absorption carried out at 37°C. for half an hour. After centrifugation the clear supernatant diluted serum was removed and preliminary precipitin tests made. Since precipitates were no longer obtained with heterologous antigens, the absorption was considered complete. Table III shows the precipitin tests with a number of homologous and

TABLE III.

Absorption Experiment: Precipitin Reactions with Serum from Rabbit Q309, Immunized with Strain S23, Type S23.

Antigen: HCl extract		Not absorbed	Absorbed with heterologous Strain S60
Strain	Type		
S23	S23	+++	+++±
S65	"	++±	++
S60	S60	+	−
S43	"	++	−
S128	"	++	−
S4	"	++	−
S24	Unclassified	++	−
S276	"	+	−
R28	"	++	−

heterologous HCl antigens, and the heterologous absorbed serum, also the control unabsorbed serum. 0.2 cc. of serum dilution (equivalent to 0.07 cc. of undiluted serum), and 0.2 cc. of antigen were mixed, and incubated in a 37°C. water bath for 2 hours. Readings were made after an additional 18 hours in the ice box. All necessary controls were negative.

Table III shows that the serum absorbed with a heterologous strain had become, in effect, a type-specific serum. This absorbed serum reacted only with antigens of the type used in immunization, while reactions with heterologous antigens were all completely negative. The heterologous strain had, therefore, not only absorbed the anti-

bodies for all strains of its own type but also the antibodies for other heterologous strains. The control lot of unabsorbed serum gave good reactions with most of these antigens. Absorption of the same serum with the homologous strain removed the antibodies for the homologous antigen also. In view of these results it was evident that Strain S24 did not belong to *Type* S23, as might have been supposed from

TABLE IV.

Absorption Experiment: Precipitin Reactions with Serum from Rabbit Q612, Immunized with Strain S23, Type S23.

Antigen: HCl extract		Not absorbed	Absorbed with hemolytic streptococcus of				
			Homologous type:	Heterologous type:			
Strain	Type		S65, Type S23	S128, Type S60	S144, Type S3	S24, unclassified	New York 5, scarlatinal
S23	S23	++±	−	++	++	++±	++±
S65	"	+++	−	+++	+++	++	++
S39	"	+++	−	++±	+++	+++	+++
S60	S60	++	−	−	−	−	−
S6	"	+±	−	−	−	−	−
S128	"	++	−	−	−	0	0
S4	"	++	−	−	−	−	±
S43	"	++	−	−	−	−	−
S72	"	++	−	−	−	0	0
S3	S3	+	−	−	−	−	−
S80	"	+	−	−	−	−	−
S144	"	+	−	−	−	−	−
S149	"	+	−	−	−	−	−
S24	Unclassified	++±	−	−	−	−	−
S276	"	±	−	−	−	0	0

the heavy precipitation with unabsorbed serum from Rabbit Q609 (Table II).

Numerous similar absorption experiments were performed with different sera and with different hemolytic streptococci as absorbing agents; essentially similar results were obtained in all these experiments. Titration of absorbed serum with varying dilutions of heterol-

ogous antigens showed that shifting of the prozone was not responsible for the negative results. Finally, a more comprehensive experiment was performed.

Experiment 4.—Five aliquot portions of serum from Rabbit Q612 were absorbed respectively with five different strains of *Streptococcus hæmolyticus*, and a sixth portion kept as an unabsorbed control. Only one strain was the type homologous

TABLE V.

Absorption Experiment: Precipitin Reactions with Serum from Rabbit Q867, Immunized with Strain S60, Type S60.

Antigen: HCl extract		Not absorbed	Absorbed with hemolytic streptococcus of				
			Homologous type:	Heterologous type:			
Strain	Type		S128, Type S60	S65, Type S23	S144, Type S3	S24, unclassified	New York 5, scarlatinal
S23	S23	+	−	−	−	−	−
S65	"	+±	−	−	−	−	−
S39	"	+	−	−	−	−	−
S60	S60	++±	−	++±	++±	++	+++
S6	"	++	±	++	++	++	++±
S128	"	++	−	−	+	0	+
S4	"	++	−	+	+±	+±	++
S43	"	+++	−	++	++	++	++±
S72	"	++±	−	+	++	0	++
S3	S3	±	−	−	−	−	±
S80	"	+	−	−	−	−	±
S144	"	+±	−	−	−	−	−
S149	"	+	−	−	−	−	−
S24	Unclassified	+	−	−	−	−	±
S276	"	+±	−	−	−	0	−

to the serum; three others were known heterologous types; the fifth belonged to an unclassified group. Absorption was accomplished with one or two 1 hour incubations, and was proved to be complete by preliminary testing with HCl extracts from heterologous strains. After being completely absorbed these sera were tested with HCl extracts from a number or strains representing different types (see Table IV). Readings with the absorbed sera made after 2 hours incubation at 37°C. and 18 hours in the ice box agreed. Controls of serum and antigen alone, and of antigen with normal serum, were negative.

A similar absorption experiment was performed with serum from Rabbit Q867, immunized with another type (see Table V).

This experiment showed again that unabsorbed antibacterial sera reacted with most antigens made from heterologous strains, although they reacted more strongly with homologous antigens than with heterologous. Absorption with bacteria of the homologous strain, or with bacteria of any strain of the same type, removed all antibodies; while absorption with any heterologous strain removed antibodies for *all* types of heterologous hemolytic streptococci, but left the type-specific antibodies practically intact. By this method, therefore, it was possible to prepare a serum which contained only type-specific antibodies.

Certain technical difficulties were encountered in these absorption experiments: complete absorption was often hard to attain because the serum could not be much diluted if it were to be used subsequently for satisfactory precipitin tests. Non-type-specific antibodies were absorbed more readily by certain strains than by others. Strains of the homologous type were more efficient in this respect than heterologous strains; and heterologous strains varied somewhat among themselves. Of the heterologous strains used in these experiments, S24 was the most effective absorbing agent. Various other workers have observed that some strains of bacteria are better than others for absorbing antibodies; and Krumwiede, Cooper, and Prevost (12) point out, in their comprehensive paper on agglutinin absorption, that this is often true. The present experiments with hemolytic streptococci indicate also that few absorptions with heavy emulsions are preferable to often repeated ones with fewer organisms. This is due partly to unknown factors, but can be partially explained by the additional dilution occasioned by using wet bacteria and also by the prolonged heating involved in repeated absorptions. Numerous absorptions with heterologous strains also tend eventually to reduce somewhat the titer of the type-specific antibodies. This may be due to a non-specific adsorption, such as occurs with kaolin, rather than to a lack of immunological specificity in the absorption process.

Examples of such overabsorption were usually found in sera which contained large amounts of non-type-specific antibody and conse-

quently required excessive absorption for its removal. Even such repeatedly absorbed sera still reacted, though in less degree, with most homologous antigens and not at all with heterologous. Such instances emphasize the importance of selecting sera with as little non-type-specific antibody as possible as well as the necessity of proper selection of the absorbing strain.

From three strains it was impossible to obtain a type-specific fraction by any of the methods which were usually successful. In one instance this was associated with loss of agglutinability; although in two others the strains were still agglutinable. While these were all old laboratory strains, so also were most of the other strains used in these experiments. It is possible that certain strains never possessed the function of producing a type-specific antigen; but it seems more probable, especially in view of the earlier successful classification of these strains, that some such factors as length of time under cultivation caused this condition. A study of other known groups of hemolytic streptococci might furnish a solution of this question.

DISCUSSION.

A type-specific substance was detected in HCl extracts of hemolytic streptococci by the absorption method. Such extracts also contained non-type-specific substances which gave confusing cross-reactions in the precipitin test when unabsorbed immune serum was used. Homologous antigens usually formed heavy flocculent precipitates as soon as they were mixed with the serum; while heterologous antigens often formed no precipitate until after the tubes had been in the ice box overnight. These non-type-specific precipitates were discs like those characteristic of type-specific carbohydrates of other species. Occasionally, however, non-type-specific precipitates appeared early and had the same flocculent characteristics as those formed by homologous antigens. Such results precluded the possibility of obtaining reliable type-specific precipitin reactions unless either the non-type-specific substances could be removed from the antigen or the non-type-specific antibodies from the serum.

While complete purification of the antigen by fractional precipitation proved extremely difficult or impossible, the preparation of type-specific serum by absorption was found to be easy. Although absorp-

tion with any strain of the homologous type removed all antibodies from the serum, absorption with bacteria from heterologous strains of hemolytic streptococci removed only the non-type-specific antibodies with the result that the serum no longer gave cross-precipitations with extracts from any heterologous strain of hemolytic streptococcus but still reacted with extracts from homologous strains, usually with only slight change in intensity. Numerous absorptions with heterologous bacteria eventually reduced the titer of type-specific antibodies; but it is probable that this is a non-specific adsorption, in the category of adsorption of antibodies by substances like kaolin, rather than an invalidation of the other experiments reported. Even in these extreme instances, however, type-specific reactions were still obtained with the absorbed serum. It was possible, therefore, to prepare antibacterial sera which contained only type-specific antibodies and consequently gave only type-specific precipitin reactions.

Of nineteen strains of hemolytic streptococcus studied, ten yielded type-specific antigens, six were tested for non-type specific antigens only since no homologous serum was available in these instances, and three failed to produce type-specific antigens. It seems probable that this failure was associated with long cultivation in the laboratory, or with other unknown factors, since these strains had been classified several years before by other methods. The classification of all other strains by the method described here agreed with that previously determined for these strains by agglutination and protection tests.

Obviously this has distinct advantages for classification of hemolytic streptococci over that possessed by the agglutination reaction. So many strains of hemolytic streptococci agglutinate spontaneously that the only methods previously applicable to their grouping were agglutinin absorption or serum protection of animals with which they were inoculated. Both methods are costly in time and material. Moreover, protection tests with strains of low virulence are impossible or inconclusive. The complexity of the antigenic structure of certain hemolytic streptococci makes the interpretation of results of agglutinin absorption at times very difficult, as shown recently by several authors (13). The application of this method to such groups might reveal a type-specific element in these strains, whereas the agglutinin absorption method has failed to indicate sharply defined types but has led to the suggestion by some of these authors of an antigenic mosaic.

The precipitin test, in contrast with the agglutination and protection tests, is applicable to any strain. The HCl extract can be prepared directly from the sediment of broth cultures in a few hours; and even when requiring additional concentration this can be completed in a day or two. The absorption of the non-type-specific antibody from the serum is the only time-consuming part of the method. Once absorption is complete, however, the sera may be kept in the ice box for months and used as required. The facility with which certain strains absorb non-type-specific antibodies is noteworthy and makes desirable the conservation of these types for this special purpose.

SUMMARY.

1. Hydrochloric acid extracts of *Streptococcus hæmolyticus* contain type-specific, as well as non-type-specific, substances. The precipitates formed by these crude extracts with homologous antibacterial serum are flocculent, while those obtained with heterologous serum are usually disc-like.

2. The type-specific substance may be detected by the use of antibacterial sera absorbed with heterologous strains of hemolytic streptococci. Such absorbed sera are type-specific: they are precipitated only by extracts of strains of the homologous type.

3. Any heterologous strain of hemolytic streptococcus absorbs the antibodies for all other heterologous strains, but homologous strains absorb type-specific antibodies as well. Numerous repeated absorptions with heterologous hemolytic streptococci tend to lower the titer of the type-specific antibody. A possible explanation of this fact is suggested.

4. Three strains did not yield a type-specific substance; and it seems probable that they had lost this function because of long continued cultivation in artificial media.

5. Classification based on the precipitin test with absorbed serum agrees with that previously determined by agglutination and protection tests. The method is, therefore, applicable to the problem of classification of the hemolytic streptococci.

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