A SEROLOGICAL DIFFERENTIATION OF SPECIFIC TYPES OF BOVINE HEMOLYTIC STREPTOCOCCI (GROUP B)

By REBECCA C. LANCEFIELD, PH.D.

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

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In a preceding paper (1) it was shown that hemolytic streptococci isolated from man, lower animals, and dairy sources can be differentiated into serological groups, which correspond, in general, to those described by other investigators on the basis of cultural and biochemical characteristics. The experimental evidence indicates that the so called C substances, upon which the group specificity depends, belong to the general class of carbohydrates, and that in each group this determinative substance is chemically distinct and serologically specific. In the particular series of strains on which this investigation was based the serological groups were found to bear a certain relationship to the origin of the cultures: Group A is composed largely of strains of human origin; Group B of those derived from mastitis in cows and from normal milk; Group C of those from various lower animals, including a number from cattle; Group D comprises in this series hemolytic streptococci from cheese; and Group E is made up of a few strains isolated from certified milk. Probably additional groups exist.

In still earlier work (2) the occurrence of serological types within Group A was determined by means of agglutination reactions combined with mouse protection tests. Later (3) it was demonstrated that specific types differentiated among Group A strains by use of the precipitin reaction correspond with those previously classified by the methods of agglutination and mouse protection. In these later studies, the so called M substances, responsible for type specificity among the members of Group A, were shown to be protein in nature. This is in contrast to the fact that the so called S substances, which determine the type specificity of certain encapsulated bacteria, have usually proved to be polysaccharides. With the possibility in mind that type specificity among certain hemolytic streptococci might in some instances be dependent upon polysaccharides, a search for mucoid and encapsulated strains was undertaken. No human strains, however, were encountered containing any other type-specific substance than the protein, M. It was soon found, on the other hand, that the members of Group B, chiefly of bovine origin, fell into distinct types, the serological specificity of which was, indeed, determined by the presence of specific polysaccharides.

The purpose of the present paper is to define the serological types found in Group B hemolytic streptococci, those derived from mastitis

in cows and from normal milk, and to present the data thus far available with respect to the chemical composition and immunological properties of the type-specific polysaccharides of two of the three specific types differentiated within this group. For purposes of comparison the data are also included showing the chemical properties of the carbohydrate, C, the group specific substance common to all organisms of Group B irrespective of their type differentiation.

Differentiation of Specific Types of Group B Hemolytic Streptococci by the Method of the Precipitin Reaction

Twenty-one strains, classified serologically as members of Group B by means of the precipitin reaction, were available for the present study. Their origin and group characteristics—cultural, biochemical, and serological—have been recorded previously (1). While the existence of specific types within this group was readily apparent, the ultimate differentiation was greatly facilitated by the production and use of antisera in which the type-specific precipitins were the dominant antibodies. The following methods, while by no means infallible, were of assistance in accomplishing this classification.

Methods

1. Immunization of Rabbits.—The most satisfactory method for inducing antibodies against polysaccharides was that of intravenous injection of rabbits with formalinized cultures. The cultures were prepared by resuspending the bacterial cells from 16 hour broth cultures in 0.85 per cent NaCl solution containing 0.2 per cent formalin. The volume was 1/20 that of the original culture. After 48 hours in the ice box, the bacterial suspensions were usually sterile. Immediately before use, these suspensions were diluted 20 times with physiological salt solution; then repeated courses of 1 cc. amounts were injected intravenously into rabbits daily for a week, followed by a week's rest. The antibody response depended somewhat upon the total dosage and the number of series of injections given. Thus, after one series of injections, the precipitin for the group-specific polysaccharide, C, was often present almost to the exclusion of other antibodies. After the second or third series of injections with the same dosage as the first, the concentration of the group-specific anti-C precipitin increased and the type-specific antibody began to appear. Thereafter, the anti-C precipitins frequently decreased in concentration or even disappeared completely, while the type-specific anti-S precipitins usually increased. Sometimes, however, one or the other of these antibodies was markedly predominant in the serum throughout the course of the immunization, while in other instances the titer of both antibodies might be

equally high or low. In some of the rabbits which showed a poor antibody response, the dosage was increased after four or five courses of injections to as much as 20 times the original amount. After this prolonged and intensive immunization, some rabbits finally developed precipitins in their sera for extracts of the homologous organism; but these antibodies proved to be not entirely type-specific, although they tended to show a certain degree of strain specificity. They were, moreover, antibodies for protein substances rather than for polysaccharides. The precipitin tests with this kind of antiserum were confusing, since the predominant reaction appeared to indicate a strain specificity dependent upon a protein. The exact immunological relationship of this protein to the other protein constituents of the cell could not be determined, even though numerous absorption experiments were performed for the purpose. For type differentiation, therefore, it was found desirable not to resort to large doses of formalinized culture in the immunization of rabbits but to use small doses in repeated series of injections. In every case it was essential to determine as far as possible, the kinds and relative concentrations of antibodies present in a given serum before employing it for classification of hemolytic streptococci.

2. Specific Absorption of Antisera.—In the experiments here reported it was possible to use the direct precipitin test for differentiating the hemolytic streptococci of Group B into specific types. This was accomplished by employing sera containing a high titer of anti-S precipitins and low titers of anti-C precipitins and antiprotein precipitins of undetermined specificity. When purely type-specific antisera were lacking, however, the following method was employed. Sera containing a mixture of antibodies of relatively high titers were absorbed with heat-killed streptococci belonging to Group B but heterologous in type to the strain used in preparing the serum. Control serum was absorbed with the homologous strain. The bacteria, centrifuged from broth cultures, were suspended in physiological salt solution and heated at 56°C. for 1 hour. The centrifuged organisms were mixed with an equal volume of undiluted serum. After 30 minutes' incubation at 37°C., the serum was separated from the bacteria by centrifugation and tested for heterologous precipitins. If absorption was incomplete, the process was repeated. It was thus possible to study the antibody content of sera and to determine certain relationships between individual strains.

3. Preparation of Extracts.—The extracts were prepared by heating the bacteria suspended in N/20 HCl as previously described (1). This crude extract was used in some instances, but in others, further separation and concentration of the active substances was accomplished by fractional precipitation with alcohol. After the addition of two to three volumes of 95 per cent ethyl alcohol, a precipitate appeared which was separated and, on resolution, was found to contain most of the type-specific S substance, together with more or less irrelevant protein material. The confusion arising in the serological relationships of these proteins was avoided as much as possible by using antisera with as little antiprotein precipitin as possible.

The supernatant alcoholic fluid, which was removed from the precipitated S substance, was concentrated on the steam bath and was found to contain most of the group-specific carbohydrate, C.

4. Digestion with Trypsin.—To test the effect of trypsin on the serologically active substances extracted from the bacteria, the following technique was employed. A 2 per cent suspension of Fairchild's trypsin in physiological salt solution was incubated for 10 minutes in a water bath at 37°C. After centrifugation, the supernatant fluid was made alkaline to phenolphthalein. Part of the enzyme solution was inactivated by heating for 10 minutes in a boiling water bath. Equal parts of the active and inactive solutions were mixed respectively with equal parts of the bacterial extract. If necessary, toluene and chloroform were added as preservatives. The mixtures were incubated for periods varying from 10 minutes to 6 days. Samples were removed at intervals and the trypsin inactivated by heating for 10 minutes in a boiling water bath. Precipitin tests were made on these samples to determine whether the serologically active substances originally present in the extracts were undigested. The activity of the trypsin was insured by proving its ability to digest a known type-specific protein, M, of a Group A strain (4). Fresh trypsin was added if shown necessary, and the digestion was continued as long as desired.

5. The Precipitin Test.—To serial dilutions of the extract, a constant volume of 0.2 cc. of undiluted serum was added and layered. Usually, tubes were set up containing 0.4 cc., 0.1 cc., and 0.025 cc. of extract in a volume of 0.4 cc. Controls with physiological salt solution plus serum, and with extract plus normal serum were included. After 20 minutes in a water bath at 37°C., the mixtures were observed for ring formation, then shaken and reincubated. Preliminary readings were made after 2 hours in the water bath, and final readings after standing overnight in the ice box.

The results of the precipitin reactions with crude hydrochloric acid extracts are given in Table I. The data recorded in the last three columns show that all 21 strains are members of Group B, as indicated by their positive reactions with an antiserum in which the group, or anti-C, precipitin, is predominant. The results recorded in Columns 2 to 10 show the serologically specific types into which these organisms have been subdivided by the use of antisera containing the type-specific, or anti-S, precipitin as the dominant immune body. Using unabsorbed antisera, all except three of the 21 strains of hemolytic streptococci studied were differentiated serologically into three sharply defined and specific types: four strains in Type I, eight in Type II, and six in Type III. Extracts of most strains of a given type reacted specifically with an antiserum of the homologous type, and gave little or no cross-reaction with immune sera of the heterologous types. Usually, in fact, no reaction was observed in heterologous sera at the end of the incubation period, and evidence of slight cross-precipitation

TABLE I

Precipitin Reactions of Group B Hemolytic Streptococci to Show the Specific Types into Which They Are Classified

Extract from	Specific type antisera (anti-S), unabsorbed									Group B antiserum (anti-C), 0.2 cc.		
	Type I, 0.2 cc.			Type II, 0.2 cc.			Type III, 0.2 cc.					
	Extract, *cc.*											
	0.4	0.1	0.025	0.4	0.1	0.025	0.4	0.1	0.025	0.4	0.1	0.025
Type I strains												
(1) O 90	++++	++++	++±	−	−	−	+	±	+	+++	+++	+++
(2) K 158A	++++	+++	+±	±	±	−	+	±	+	+++	+++	+++
(3) K 107	++++	+++	+±	±	±	±	−	±	+	+++	+++	++±
(4) K 127	*+	+	±	±	±	±	±	±	−	+	+++	+++
Type II strains												
(1) V 8	−	−	±	+++±	+++±	++	±	±	0	+++±	+++	+++
(2) V 9	−	−	±	+++±	+++	++	+	±	±	+	+++	+++
(3) C 69	−	−	±	+++±	+++	++	±	±	0	++	+++	++±
(4) B 92	−	−	±	++++	++±	+	±	−	+	+++±	+++	+++
(5) B 112	−	−	±	++++	+++	+	+	±	0	+++	+++	+++±
(6) B 116	−	−	±	+++	++	±	±	−	+	++	++±	+±
(7) B 120	−	−	±	++++	+++±	++	±	±	0	+++	+++	++
(8) B 132	−	±	±	++++	+++	+±	−	−	−	+++	+++	+±
Type III strains												
(1) M 216	−	−	±	±	−	−	+++	++±	±	+++	+++	++±
(2) K 151 A	−	±	±	±	±	−	+++	++±	+	+	+++	+±
(3) K 198	−	−	±	−	−	−	+++	++	±	++	+++	+++
(4) B 63	−	−	±	±	±	−	+++	++	+	+++	+++	+++±
(5) B 115	−	−	±	±	±	−	† ++±	+	−	+++	+++	++++
(6) B 135	−	−	−?	±	±	−	† +	±	−	+++	+++	+++±
Unclassified strains												
(1) B 125	−	±	±	±	−	−	±	−	−	+++	++++	+++
(2) B 126	−	−	±	±	±	−	±	±	−	+++	+++	+++±
(3) K 126	−	±	±	±	±	−	±	−	−	+++	+++	+++

The following signs are used in all tables: ++++ to ± indicate degrees of reaction; − indicates a negative reaction; 0 indicates that the test was not made.

Controls with normal serum plus extract, and with serum plus saline, were all negative.

* Later experiments, described in the text, showed definitely that this strain belonged in Type I. † Concentrated extracts of these two strains gave as strong reactions with Type III antisera as any of the other members of Type III.

was only detectable after the reaction mixtures had stood overnight in the ice box. In nearly all instances the unpurified hydrochloric acid extracts gave entirely unequivocal results.

The three exceptional strains (K 127 in Type I, and B 115 and B 135 in Type III) seemed to contain minimal amounts of type-specific substance as shown by the slight precipitin reactions with antisera which proved later to be of the homologous type, and by the slight amount of type-specific antibody demonstrable in the serum of rabbits immunized with these organisms. Extracts of Strains B 115 and B 135 containing the type-specific substances concentrated by precipitation with alcohol were of sufficient potency to prove by the precipitin reaction that these strains belonged in Type III. The fact that Strain K 127 belonged in Type I was indicated by the use of similarly concentrated extracts and was further verified by preparing antisera with this organism which reacted specifically with a purified preparation of a known Type I polysaccharide and afforded mice protection against other strains belonging to Type I.

Similarly, the low content of type-specific substance may account for the difficulty in classifying Strains B 125, B 126, and K 126. This deficiency was suggested by the total lack of any demonstrable type-specific antibodies in the serum of rabbits immunized with these strains.

The classification shown in Table I was confirmed in numerous experiments by specific absorption of antibodies in the manner detailed under methods. An example is given in Table II.

One portion of Serum R 36-60, from a rabbit immunized with Strain O 90, Type I, was absorbed with heat-killed bacteria from a different Type I strain (K 158 A); and a second portion with bacteria from a Type II strain (C 69); a third portion was kept as a control. In this particular experiment, the serum was diluted with equal parts of physiological salt solution. Absorption was repeated a second time. The sera were then tested with several extracts of organisms of homologous and heterologous types, as shown in Table II.

The serum had a high titer of both group- and type-specific antibodies, as shown by the reactions of the unabsorbed serum with crude extracts of strains of all three types, by the reactions with a purified preparation of type-specific S substance of the homologous Type I strain, and by the reactions with the purified group-specific C substance. Two absorptions with a strain of homologous type (K 158 A, Type I) removed all antibodies from the serum. Similar absorptions with a strain of heterologous type (C 69, Type II) removed the group-specific antibody for C, as shown by the negative reactions with the crude extracts of Type II and Type III strains, and by the negative reaction with the purified group-specific substance, C. Absorption with this heterologous Type II strain did not, however, remove the antibody for the Type I specific polysaccharide, for the absorbed serum still reacted specifically with all preparations containing the Type I S substance.

In the course of these experiments, some difficulty and irregularity was encountered in the absorption of sera having a high concentration of antibodies for protein substances. Since, however, the antisera useful for type classification did not contain much antiprotein precipitin, this difficulty is not significant in the work reported here.

The type differentiation above detailed was confirmed by passive protection tests in mice where virulent strains were available.

Only two of the 21 strains were sufficiently virulent for this purpose and these both belonged to Type I. They killed mice regularly in doses ranging from 10^{-6} cc. to 10^{-8} cc. of an 18 hour broth culture. All other strains were of such low virulence that even doses of 10^{-1} cc. did not kill mice, and in many instances 0.5 cc. was non-fatal.

TABLE II

Absorption Experiment to Confirm Type Differentiation

Extract used in precipitin reaction	Type I serum, R 36-60		
	Unabsorbed control serum	Absorbed with Strain K 158 A Type I	Absorbed with Strain C 69 Type II
Type I Strains			
(1) O 90	+++	−	++
(2) K 158 A	+++±	−	++
(3) K 107	+++	−	+±
(4) K 127	++±	−	+±
Type II Strain			
(1) B 112	++±	−	−
Type III Strain			
(1) K 198	++±	−	−
Purified substances from Strain O 90 (Type I)			
(1) Type-specific (S substance)	++±	−	++
(2) Group-specific (C substance)	++++	−	−

The mouse protection tests were performed as follows: 0.5 cc. of serum was injected intraperitoneally into each mouse; the following day, 0.5 cc. of the appropriate dilution of a 16 hour broth culture, diluted serially in broth, was also injected intraperitoneally. All mice surviving the 10 day period of observation were considered effectively protected and were recorded as survivors. Estimations of the number of organisms in a given inoculum were made by plating in blood agar 0.5 cc. amounts of the three highest dilutions of the culture, and counting the colonies after 48 hours' incubation at 37°C.

The results of the protection tests against the virulent Type I strains (O 90 and K 158 A) are shown in Table III. Type I antiserum afforded protection to mice against 10 to 100 million fatal doses of

TABLE III

Cross-Protection Tests in Mice to Confirm Type Differentiation
The Two Virulent Type I Strains Tested with Sera of Homologous and Heterologous Types

Culture	Virulence controls		Antiserum against		
	No serum	Normal serum	Strain O 90 Type I	Strain V 9 Type II	Strain M 216 Type III
cc.					
* Strain O 90 Type I					
10^{-8}	D 53 hrs.	—	—	—	—
10^{-7}	D 51 "	—	—	—	—
10^{-6}	D 48 "	D 41 hrs.	S	D 40 hrs.	D 41 hrs.
10^{-5}	D 48 "	D 41 "	S	D 65 "	D 41 "
10^{-4}	D 72 "	D 41 "	S	D 41 "	D 41 "
10^{-3}	D 21 "	D 42 "	S	D 41 "	D 23 "
10^{-2}	D 17 "	D 17 "	S	D 17 "	D 17 "
10^{-1}	D 17 "	D 17 "	S	D 17 "	D 17 "
† Strain K 158 A Type I					
10^{-8}	S	—	S	S	‡S
10^{-7}	D 42 hrs.	—	S	S	D 65 hrs.
10^{-6}	D 42 "	—	S	D 41 hrs.	S
10^{-5}	D 18 "	—	S	D 41 "	D 41 hrs.
10^{-4}	D 33 "	—	S	D 89 "	D 65 "
10^{-3}	D 18 "	—	S	D 41 "	D 160 "
10^{-2}	D 18 "	—	S	D 18 "	D 116 "
10^{-1}	D 18 "	—	S	D 17 "	D 18 "

S indicates survival for 10 days.
D indicates death within the number of hours indicated.
— indicates test omitted.
* Estimated by plate counts as 346 million colonies per cc.
† No colony counts made.
‡ For this test a Type III serum prepared against Strain B 63 was used.

cultures of the homologous type, while Type II and Type III antisera gave no protection whatever against these strains. In the control series, without serum or with normal serum, no protection was ob-

served. The Type I serum used in the experiment recorded in Table II was prepared by immunization of rabbits with Strain O 90. Similar tests with serum prepared with another strain of Type I (K 127) also resulted in protection of the mice.

It is interesting to observe that type-specific protection is effective in Group B when infecting organism and specific immune serum are injected simultaneously. With Group A organisms, in which the type-specific substance is a protein, it is necessary to inject the antiserum at least 8 hours before the infecting organism in order to demonstrate protection; but with Group B organisms, in which the type-specific substance is a polysaccharide, the simultaneous injection of the organism and its homologous antiserum results in specific protection of the mice, as is also true with certain other bacteria, such as the pneumococcus, in which a polysaccharide is the type-specific substance.

The Agglutination Reaction

The method of agglutination was used in an attempt to differentiate these Group B strains into types before the method of specific precipitation was adopted, but it was only partially successful on account of the particularly troublesome cross-agglutinations among these strains. While this difficulty might have been overcome by employing specifically absorbed sera in the agglutination reaction, it seemed more convincing, as well as easier, to use the direct precipitin test for the type classification rather than to rely entirely on the results obtained with absorbed sera. With the precipitin test, the types were sharply and unequivocally defined where certain antisera were used, although the agglutination test with these same sera yielded many confusing cross-reactions.

The agglutinations were performed by diluting the serum in infusion broth and mixing 0.5 cc. of serum dilution with 0.5 cc. of 18 hour broth culture. If the culture showed any tendency to agglutinate spontaneously, it was washed and resuspended in fresh broth before use. The tests were read after 2 hours' incubation at 56°C.

Table IV shows the results of the agglutination reactions of one strain in each type giving the most marked type-specific reaction, and of one strain giving the most marked cross-reaction. The sera were those used for type differentiation with the precipitin reaction (recorded in Table I). With most Type I strains, O 90 for example, there was very little cross-agglutination in the sera of other types,

TABLE IV

Cross-Agglutination Reactions

Culture	Antiserum against																	
	Type I					Type II							Type III					
	Final serum dilutions																	
	1-20	1-40	1-80	1-160	1-320	1-20	1-40	1-80	1-160	1-320	1-640	1-1280	1-20	1-40	1-80	1-160	1-320	1-640
Type I strains																		
(1) O 90	++++	++++	++++	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
(2) K 127	++±	++±	+±	±	−	+±	+±	+±	−	−	−	−	+±	±	−	−	−	−
Type II strains																		
(1) B 132	±	−	−	−	−	++++	++++	++++	++++	++++	+++	+++	++	+±	+±	+	−	−
(2) B 92	−	−	−	−	−	++++	+++	+++	−	−	−	−	+++	+++	++	−	−	−
Type III strains																		
(1) K 151	−	−	−	−	−	+	±	−	−	−	−	−	++++	++++	++++	+++±	++	+
(2) B 63	++	++	±	−	−	++++	++++	++++	++++	++++	++++	+++	++++	++++	++++	++++	+++	++±

Controls with broth and with normal serum in the same series of dilutions were negative.

although with such strains as K 127, containing minimal amounts of type-specific substance, considerable cross-reaction was observed. Because the Type I serum employed gave only slight cross-reactions with strains of other types, the agglutination test was of value in classifying the Type I strains. The agglutinated organisms coalesced into a compact disc, like that seen in type-specific pneumococcus agglutinations in contrast to the less compactly agglutinated mass usual in streptococcus agglutinations. A further similarity with pneumococcus agglutinations was the low agglutinin titer attained, usually about 1–80 and not often exceeding 1–320. Between Types II and III strains and sera there was a large amount of cross-reaction, so that it was impossible to classify most strains of these two types by the simple agglutination test. Thus, Strain B 132, the most specifically reacting strain of Type II, still showed considerable cross-agglutination in Type III serum. Among Type III organisms, Strain K 151 A gave specific reactions, but most other members of this type gave cross-reactions like that shown by Strain B 63. With these two types, especially Type III, the agglutinated organisms coalesced less firmly into discs than the organisms of Type I, and the agglutinin titers reached the high level commonly found among antisera for streptococci of other groups.

The sera used for these agglutinations were selected for their low content of non-type-specific antibodies, and did not give much cross-reaction when used in the precipitin test (Table I). In the agglutination reaction, however, which is a more sensitive test for antibody, even this low concentration of group antibodies was sufficient to give marked cross-reactions. Since other sera, containing a higher concentration of group antibodies gave much more cross-agglutination, this reaction seemed less well adapted than the precipitin test for the study of the classification and antigenic composition of these strains of hemolytic streptococci.

The Chemical Nature of the Substances Determining Group and Type Specificity

It may be recalled that type specificity among Group A strains depends upon bacterial proteins, the so called M substances (3). The occurrence, therefore, of type-specific carbohydrates, previously unknown among hemolytic streptococci, was of particular interest in the typing of Group B strains.

The formation of typical disc-like precipitates, so generally characteristic of the union between bacterial polysaccharides and their corresponding precipitins, was the first suggestion of the possibility that the reactive substances in this group

were carbohydrates rather than proteins. Disc formation was particularly marked with Type I strains, although physical conditions, such as the relative concentrations of the reactive substance and its antibody, and the amount of shaking which the mixture received during incubation, played an important rôle in determining the character of the disc. There was a slightly greater tendency toward the formation of coarse, loose discs with Type II strains, and only under optimal conditions did Type III strains form characteristic discs. Further investigation indicated, however, that the poorer disc formation probably resulted partly from a lower concentration of type-specific antibody, and partly from an admixture of other reactive substances, protein in nature, which combined with group antibody present in the serum. This was evident in tests made with serum from which all except the type antibody had been absorbed, after which the type-specific precipitates appeared as more characteristic discs.

Digestion experiments with trypsin also suggested the polysaccharide nature of these type-specific substances of members of Group B. Discs typical of polysaccharides still occurred after digesting the extracts with trypsin; and the type-specific reactivity of these extracts was not diminished by even 6 days of tryptic digestion.

This additional indication of the non-protein nature of the reactive substances led to the preparation and purification of the so called S substance for the purpose of chemical analysis. For comparison, the group-specific C substance, also present in the crude bacterial extracts, was separated and analyzed at the same time.

Since the culture fluids contained only small amounts of either substance, it was necessary to extract the bacteria after centrifugation from the 0.1 per cent dextrose infusion broth, in which they were grown. The bacterial sediment was extracted by heating with N/20 hydrochloric acid, as already described (1). The extract from the Type I culture, Strain O 90, was then purified by repeated precipitations with three volumes of 95 per cent ethyl alcohol. After dialyzing and drying, this preparation (O 90, S 6, in Table V) was analyzed chemically and found to be essentially of polysaccharide composition.[1] It gave negative protein tests with the biuret reaction and trichloracetic acid, but it gave a very strongly positive Molisch reaction. The total nitrogen, probably largely impurity, was 2.14 per cent; and reducing sugars, calculated as glucose, after acid hydrolysis were 76.8 per cent; the acid equivalent was 5230; the optical rotation was $-4.8°$. The substance reacted in homologous immune serum in a dilution of at least 1–3,000,000.

[1] I am greatly indebted to Dr. W. F. Goebel for much advice in the preparation of these substances, and to Dr. Goebel and Dr. F. H. Babers for all of the analyses, which they were kind enough to perform.

When the foregoing analysis was made, the existence of the carbohydrate, C, specific for all Group B hemolytic streptococci, was unknown. Following the discovery of this substance, the above preparation of Type I specific polysaccharide was retested serologically and found to be a mixture of the type-specific S substance and the group-specific C substance, as shown by the precipitin tests recorded in the last four columns of Table V.

These two substances were therefore separated from each other by fractional precipitation with alcohol of a concentrated solution of preparation O 90, S 6. The S substance was precipitated with two volumes of 95 per cent ethyl alcohol, while the C substance required a higher concentration of alcohol for complete precipitation. These two fractions were then dialyzed and dried as before. The analyses, given in Table V (O 90, S 6a, and O 90, C 6), show that both are in all probability polysaccharides. The fraction containing the S substance still gave a type-specific precipitin test in a dilution of at least 1–3,000,000, but no longer reacted with serum containing the group antibody. The fraction containing the C substance, on the contrary, gave no reaction with purely type-specific antisera, but gave a specific precipitate in a dilution of at least 1–4,000,000 with antisera rich in group-specific antibody.

The material from the Type II strain, V 9, was similarly extracted.

One lot, prepared by repeated precipitation with alcohol, yielded a small amount of very impure S substance; although the C substance, collected from the same lot, was more successfully purified. Its characteristics are recorded in Table V (V 9, C 1). A second lot of S substance was collected from Strain V 9 and purified by precipitating the impurities with trichloracetic acid, as suggested by Goebel (5). The S and C substances, freed of protein by this method, were then separated by fractional precipitation with ethyl alcohol. The analysis of the type-specific S substance from this preparation is given in Table V (V 9, S 3).

The data presented in Table V indicate fairly clearly that the group-specific C substance and the type-specific S substances of Types I and II are carbohydrate in nature. Due to the great difficulty in obtaining more than very small amounts of these substances, fuller chemical data are not available. For the same reason, no analysis was made of the S substance characteristic of Type III strains.

All of the S and C preparations analyzed (Table V) gave negative qualitative protein tests; most of them showed a total nitrogen content of approximately 2 per cent, part of which may be due to impurities. All gave very strong Molisch

TABLE V

Properties of Type-Specific S Substances and of Group-Specific C Substance

Preparation			Chemical analysis		Serological reactions			
					Highest dilution of substance reacting in the precipitin test with			
					*Anti-S serum			*Anti-C serum
Strain	Lot No.	Type or group substance	Total nitrogen	Reducing sugars after hydrolysis (calculated as glucose)	Type I	Type II	Type III	Group B
			per cent	*per cent*				
O 90	† S 6	Mixture: S of Type I and C of Group B	2.14	76.8	1–3,000,000	—	—	1–100,000
O 90	S 6a	S of Type I	1.93	65.6	1–3,000,000	—	—	—
O 90	C 6	C of Group B	‡Not done	61.0	—	—	—	1–4,000,000
V 9	S 3	S of Type II	1.43	61.2	—	1–4,000,000	—	—
V 9	C 1	C of Group B	2.34	39.7	—	—	—	1–4,000,000

— indicates negative reaction.

* These were all antibacterial sera. Certain antisera rich in S antibodies and poor in C antibodies, were used for anti-S sera. Other antisera, rich in C antibodies, were used in titrating the C substances.

† This preparation proved to be a mixture consisting chiefly of type-specific S substance but containing an appreciable quantity of group-specific C substance. These substances were separated and analyzed as O 90, S 6a, and O 90, C 6.

‡ Insufficient material for this analysis. A 1–265 dilution was biuret-negative and was not precipitated by picric, trichloracetic, or sulfosalicylic acids. It gave a very strong Molisch reaction.

reactions, even in considerable dilution; and after acid hydrolysis the reducing sugars, calculated as glucose, varied from 40 to 77 per cent in the different preparations. The differences in the group-specific C substances in preparations from the strains representing Types I and II were presumably due to the presence of impurities, for, serologically, these two preparations were identical in their reactions. The S preparations, on the contrary, were serologically distinct in each type and reacted only with antisera of the homologous type.

To summarize, the chemical analyses, together with the non-digestibility with trypsin, and the formation of disc-like precipitates, seem sufficient evidence to characterize as polysaccharides the group-specific C substance and the type-specific S substances (Types I and II) isolated from members of Group B hemolytic streptococci.

DISCUSSION

The data presented show that the hemolytic streptococci, differentiated into Group B by the serological methods described in a preceding paper (1) can be further subdivided into specific types. Although the agglutination reaction was found unsuitable for differentiation of types on account of the large amount of cross-agglutination, the precipitin reaction proved suitable for this classification under proper precautions. The principle involved was the production of immune sera in which the type-specific antibody was predominant. This was achieved, to a certain extent, by the particular method of immunization and also by the method of selective absorption of the antisera. Type-specific antisera thus prepared were used with bacterial extracts in the precipitin reaction to differentiate into specific types the 21 strains of Group B hemolytic streptococci which were available for this study. With three exceptions, these strains fell into three specific types. Passive protection tests in mice furnished confirmatory evidence of this specific type classification.

The purified type-specific substances obtained from bacterial extracts of representatives of Types I and II were analyzed chemically, and even though the small amount of available material precluded full chemical analyses, the data indicate that the type-specific substances belong to the class of carbohydrates. The evidence for their non-protein nature is further supported by the complete failure of trypsin to destroy them. Although a chemical analysis of the Type III

specific substance was not made on account of the difficulty of obtaining material, its resistance to prolonged tryptic digestion was considered sufficiently conclusive evidence of its non-protein nature.

For purposes of comparison with these type-specific polysaccharides (designated as S substances), the chemical analysis is also given of the substance (designated as C) which determines the group specificity of all members of Group B irrespective of type. As previously shown (1), substances identical in their serological reactions are obtainable from all members of Group B; and the data, while insufficient to establish the chemical identity of the C substances extracted from different strains, are, nevertheless, sufficient to show that the C substance in the different preparations is in every case of carbohydrate nature. Each member of Group B hemolytic streptococci contains, therefore, one polysaccharide, C, which determines its group specificity, and another polysaccharide, S, which determines its type specificity.

The recent work of Stableforth (6) also demonstrated specific types among streptococci of bovine origin. The strains studied by him were classified into groups on the basis of cultural and biochemical tests. The group containing all of the hemolytic strains in his collection also included a large number of non-hemolytic strains. He suggests, on the basis of certain observations made in the course of his work, the possibility of a change in hemolytic properties, but reserves the further consideration of such a possibility until more evidence has accumulated.[2] With the exception of the non-hemolytic strains, the biochemical and cultural properties of the streptococci designated by Stableforth as "Group I" correspond to those here included in Group B. Using the precipitin reaction, as well as Griffith's slide agglutination technique (7), with both absorbed and unabsorbed sera, Stableforth was able to classify the 91 strains of "Group I" into three distinct serological types. It is not known whether these types correspond to those found among the Group B strains studied here.

Stableforth's comprehensive study was not concerned with the chemical nature of the determinative type-specific substances, and he supposed, on account of the character of the precipitates formed at 50–55°C., that the type-specific substances were protein in nature, and similar to the type-specific substances of hemolytic streptococci of human origin. In view of the present study, it seems more probable, however, that the non-disc-like character of the type-specific precipitates observed by Stableforth was due, rather to physical factors, such as the relative concentrations of the reactive substance and antibody, or possibly the higher tem-

[2] A report of a change in the hemolytic properties of one Group B strain included in the present studies will be the subject of a subsequent paper.

perature used in incubation, than to the presence of type-specific substances of protein nature.

A summary of the differentiation of hemolytic streptococci, thus far achieved, is given in Table VI, which also contains the results of the study of the chemical nature of the determinative substances in so far

TABLE VI

Summary of the Serological Differentiation of Hemolytic Streptococci into Groups and Types

Hemolytic streptococci differentiated into groups by precipitation with group-specific substance, C, a polysaccharide	Chief source	No. of strains studied	Type specificity within the group determined by
Group A	Man	23*	Precipitation with type-specific protein, M, agglutination, and mouse protection. Many types
Group B	Cattle	21	Precipitation with type-specific polysaccharide, S; and in some instances agglutination and mouse protection. 3 types include all but 3 of these strains
Group C	Various animals: cattle, guinea pigs, rabbits, horses, swine, chickens, foxes	49	Not well studied
Group D	Cheese	8	" " "
Group E	Certified milk	3	
Unclassified	Man, cattle	2	

* Many more strains of human origin have been tested and found positive for the presence of the C substance characteristic of Group A, but only 23 were used in the particular study (1) in which the group classification was made. Other Group A strains have also been classified as to type by the precipitin method (3, 8, 9).

as they are now known. It is evident that there exist groups (1) having more or less common animal origins, and that each group elaborates a carbohydrate substance (C) upon which the primary classification depends. The chemical nature of the substances responsible in any particular group for the further division into types is unpredictable and can only be determined experimentally. Previ-

ously (3) it was established that the serological differentiation into types among the members of Group A is determined by a protein (M). The present work, on the other hand, shows that the types existing among members of Group B are differentiated by carbohydrates specific for each type.

SUMMARY

1. Hemolytic streptococci of Group B (derived chiefly from cattle) have been further subdivided by use of the precipitin reaction into specific types.

2. With three exceptions, the 21 strains of Group B were differenated into three specific types.

3. Chemical analyses of the type-specific substances of Group B strains of Types I and II show that they are polysaccharides (S substances). This is in contrast to the fact that proteins (M substances) were previously shown to determine type specificity among strains of human origin (Group A).

4. The group-specific substance, C, serologically identical in all members of Group B, was also identified as of polysaccharide nature.

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